PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [5]:
C12N 1/21, 9/88, 15/31, 15/60, 15/61

A1

(11) International Publication Number: **WO 94/25567**

(43) International Publication Date: 10 November 1994 (10.11.94)

(21) International Application Number: PCT/US94/04495

(22) International Filing Date: 22 April 1994 (22.04.94)

(30) Priority Data:

08/052,206	23 April 1993 (23.04.93)	US
08/053,615	23 April 1993 (23.04.93)	US

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

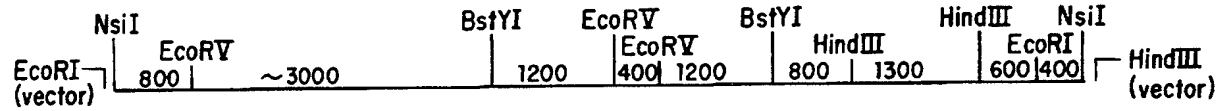
Published
With international search report.

(71) Applicant: AMERICAN CYANAMID COMPANY [US/US]; One Cyanamid Plaza, Wayne, NJ 07470 (US).

(72) Inventors: RYAN, Michael, Joseph; 34 Northwood Drive, West Milford, NJ 07480 (US). KHANDKE, Kiran, Manohar; 120 East Allison Avenue, Nanuet, NY 10954 (US). TILLEY, Bruce, Clifford; RR2, Box 27, Cresco, PA 18326 (US). LOTVIN, Jason, Arnold; 985 Roosevelt Avenue, Union, NJ 07083 (US).

(74) Agent: GORDON, Alan, M.; American Cyanamid Company, One Cyanamid Plaza, Wayne, NJ 07470 (US).

(54) Title: CLONING AND EXPRESSION OF THE CHONDROITINASE I AND II GENES FROM *P. VULGARIS*



(57) Abstract

This invention relates to the DNA sequence encoding the major protein component of chondroitinase ABC, which is referred to as "chondroitinase I", from *Proteus vulgaris* (*P. vulgaris*), which is contained in the Nsi fragment shown in the figure. This invention further relates to the DNA sequence encoding a second protein component of chondroitinase ABC, which is referred to as "chondroitinase II", from *P. vulgaris*, to the cloning and expression of the genes containing these DNA sequences, to the amino acid sequences of the recombinant chondroitinase I and II, and to methods for the isolation and purification of recombinant chondroitinase I or II. These methods provide significantly higher yields and purity than those obtained by adapting for the recombinant enzymes the method previously used for isolating and purifying native chondroitinase I enzyme from *P. vulgaris*.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

CLONING AND EXPRESSION OF THE CHONDROITINASE I AND II GENES FROM P. VULGARIS

Field of the Invention

This invention relates to the DNA sequence encoding the major protein component of chondroitinase ABC, which is referred to as "chondroitinase I", from Proteus vulgaris (P. vulgaris). This invention further relates to the DNA sequence encoding a second protein component of chondroitinase ABC, which is referred to as "chondroitinase II", from P. vulgaris. This invention also relates to the cloning and expression of the genes containing these DNA sequences and to the amino acid sequences of the recombinant chondroitinase I and II enzymes encoded by these DNA sequences.

This invention additionally relates to methods for the isolation and purification of the recombinantly expressed major protein component of chondroitinase ABC, which is referred to as "chondroitinase I", from Proteus vulgaris (P. vulgaris). This invention further relates to methods for the isolation and purification of the recombinantly expressed second protein component of chondroitinase ABC, which is referred to as "chondroitinase II", from P. vulgaris. These methods provide significantly higher yields and purity than those obtained by adapting for the recombinant enzymes the method previously used for isolating and purifying the native chondroitinase I enzyme from P. vulgaris.

Background of the Invention

Chondroitinases are enzymes of bacterial origin which have been described as having value in dissolving the cartilage of herniated discs without disturbing the stabilizing collagen components of those discs.

Examples of chondroitinase enzymes are chondroitinase ABC, which is produced by the bacterium P. vulgaris, and chondroitinase AC, which is produced by A. aurescens. The chondroitinases function by degrading polysaccharide side chains in protein-polysaccharide complexes, without degrading the protein core.

Yamagata et al. describes the purification of the enzyme chondroitinase ABC from extracts of P. vulgaris (Bibliography entry 1). The enzyme selectively degrades the glycosaminoglycans chondroitin-4-sulfate, dermatan sulfate and chondroitin-6-sulfate (also referred to respectively as chondroitin sulfates A, B and C) at pH 8 at higher rates than chondroitin or hyaluronic acid. However, the enzyme did not attack keratosulfate, heparin or heparitin sulfate.

Kikuchi et al. describes the purification of glycosaminoglycan degrading enzymes, such as chondroitinase ABC, by fractionating the enzymes by adsorbing a solution containing the enzymes onto an insoluble sulfated polysaccharide carrier and then desorbing the individual enzymes from the carrier (2).

Brown describes a method for treating intervertebral disc displacement in mammals, including humans, by injecting into the intervertebral disc space effective amounts of a solution containing chondroitinase ABC (3). The chondroitinase ABC was

isolated and purified from extracts of P. vulgaris. This native enzyme material functioned to dissolve cartilage, such as herniated spinal discs. Specifically, the enzyme causes the selective chemonucleolysis of the nucleus pulposus which contains proteoglycans and randomly dispersed collagen fibers.

Hageman describes an ophthalmic vitrectomy method for selectively and completely disinserting the ocular vitreous body, epiretinal membranes or fibrocellular membranes from the neural retina, ciliary epithelium and posterior lens surface of the mammalian eye as an adjunct to vitrectomy, by administering to the eye an effective amount of an enzyme which disrupts or degrades chondroitin sulfate proteoglycan localized specifically to sites of vitreoretinal adhesion and thereby permit complete disinsertion of said vitreous body and/or epiretinal membranes (4). The enzyme can be a protease-free glycosaminoglycanase, such as chondroitinase ABC. Hageman utilized chondroitinase ABC obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan.

In isolating and purifying the chondroitinase ABC enzyme from the Seikagaku Kogyo material, it was noted that there was a correlation between effective preparations of the chondroitinase in vitrectomy procedures and the presence of a second protein having an apparent molecular weight (by SDS-PAGE) slightly greater than that of the major protein component of chondroitinase ABC. The second protein is now designated "chondroitinase II", while the major protein component of chondroitinase ABC is referred to as "chondroitinase I." The chondroitinase I and II proteins are basic proteins at neutral pH, with similar isoelectric points of 8.30-8.45. Separate

purification of the chondroitinase I and II forms of the native enzyme revealed that it was the combination of the two proteins that was active in the surgical vitrectomy rather than either of the proteins individually.

Use of the chondroitinase I and II forms of the native enzyme to date has been limited by the small amounts of enzymes obtained from native sources. The production and purification of the native forms of the enzyme has been carried out using fermentations of P. vulgaris in which its substrate has been used as the inducer to initiate production of these forms of the enzyme. A combination of factors, including low levels of synthesis, the cost and availability of the inducer (chondroitin sulfate), and the opportunistically pathogenic nature of P. vulgaris, has resulted in the requirement for a more efficient method of production. In addition, the native forms of the enzyme produced by conventional techniques are subject to degradation by proteases present in the bacterial extract. Therefore, there is a need for a reliable supply of pure material free of contaminants in order for the medical applications of the two forms of this enzyme to be evaluated properly and exploited. There is also a need for methods to isolate and purify a reliable supply of the chondroitinase I and II enzymes free of contaminants.

Summary of the Invention

Accordingly, it is an object of this invention to produce chondroitinase I and chondroitinase II in quantities not readily achievable using present non-recombinant bacterial fermentation and extraction techniques.

It is a further object of this invention to produce chondroitinase I and chondroitinase II, each in a form substantially free of proteases which would otherwise degrade the enzyme and cause a loss of its activity.

These objects are achieved through the use of an alternative approach to the problems presented by large scale bacterial fermentation of these two forms of the enzyme. Separately for chondroitinase I and chondroitinase II, the gene that encodes the enzyme is cloned and the enzyme is expressed at high levels in a heterologous host. In a preferred embodiment, this invention is directed to the cloning of the P. vulgaris gene for chondroitinase I and the high level expression of that enzyme in E. coli, as well as the cloning of the P. vulgaris gene for chondroitinase II and the high level expression of that enzyme in E. coli.

This invention provides a purified isolated DNA fragment of P. vulgaris which comprises a sequence encoding for chondroitinase I. This invention further provides a purified isolated DNA fragment of P. vulgaris which hybridizes with a nucleic acid sequence encoding for amino acids as follows:

(a) the chondroitinase I enzyme with its signal peptide (SEQ ID NO:2, amino acids 1-1021) or a biological equivalent thereof (encoded for example by: (1) nucleotides numbered 119-3181 of SEQ ID NO:1, and (2) nucleotides numbered 119-3181 of SEQ ID NO:3, where the three nucleotides immediately upstream of the initiation codon are changed (SEQ ID NO:3, nucleotides 116-118));

(b) the mature chondroitinase I enzyme (SEQ ID NO:2, amino acids 25-1021) or a biological equivalent thereof (encoded for example by: (1) nucleotides numbered 191-3181 of SEQ ID NO:1, and (2) nucleotides numbered 191-3181 of SEQ ID NO:3, where the three nucleotides immediately upstream of the initiation codon are changed (SEQ ID NO:3, nucleotides 116-118)); and

(c) the mature chondroitinase I enzyme where the sequence encoding the signal peptide has been replaced with a sequence which adds a methionine residue to the amino terminus of the enzyme (SEQ ID NO:5, amino acids 24-1021) or a biological equivalent thereof (encoded for example by nucleotides numbered 188-3181 of SEQ ID NO:4).

The recombinant chondroitinase I is produced by transforming a host cell with a plasmid containing a purified isolated DNA fragment of P. vulgaris which contains one of the above-described sequences, and culturing the host cell under conditions which permit expression of the enzyme by the host cell.

This invention also provides a purified isolated DNA fragment of P. vulgaris which comprises a sequence encoding for chondroitinase II. This invention further provides a purified isolated DNA fragment from P. vulgaris which hybridizes with a nucleic acid sequence encoding for amino acids as follows:

(a) the chondroitinase II enzyme with its signal peptide (SEQ ID NO:40, amino

acids 1-1013) or a biological equivalent thereof (encoded for example by nucleotides numbered 3238-6276 of SEQ ID NO:39); and

(b) the mature chondroitinase II enzyme (SEQ ID NO:40, amino acids 24-1013) or a biological equivalent thereof (encoded for example by nucleotides numbered 3307-6276 of SEQ ID NO:39).

The recombinant chondroitinase II is produced by transforming a host cell with a plasmid containing a purified isolated DNA fragment of P. vulgaris which contains one of the above-described sequences, and culturing the host cell under conditions which permit expression of the enzyme by the host cell.

It is an additional object of this invention to provide methods for the isolation and purification of the recombinantly expressed chondroitinase I enzyme of P. vulgaris.

It is a particular object of this invention to provide methods which result in significantly higher yields and purity of the recombinant chondroitinase I enzyme than those obtained by adapting for the recombinant enzyme the method previously used for isolating and purifying the native chondroitinase I enzyme from P. vulgaris.

These objects are achieved through either of two methods described and claimed herein for the chondroitinase I enzyme. The first method comprises the steps of:

(a) lysing by homogenization the host cells which express the recombinant chondroitinase I enzyme to release the enzyme into the supernatant;

(b) subjecting the supernatant to diafiltration to remove salts and other small molecules;

(c) passing the supernatant through an anion exchange resin-containing column;

(d) loading the eluate from step (c) to a cation exchange resin-containing column so that the enzyme in the eluate binds to the cation exchange column; and

(e) eluting the enzyme bound to the cation exchange column with a solvent capable of releasing the enzyme from the column.

In the second method, prior to step (b) of the first method just described, the following two steps are performed:

(1) treating the supernatant with an acidic solution to precipitate out the enzyme; and

(2) recovering the pellet and then dissolving it in an alkali solution to again place the enzyme in a basic environment.

It is a further object of this invention to provide methods for the isolation and purification of the recombinantly expressed chondroitinase II enzyme of P. vulgaris.

It is an additional object of this invention to provide methods which result in significantly higher yields and purity of the recombinant chondroitinase II enzyme than those obtained by adapting for the recombinant enzyme the method previously used for isolating and purifying the native chondroitinase I enzyme from P. vulgaris.

These objects are achieved through either of

two methods described and claimed herein for the chondroitinase II enzyme. The first method comprises the steps of:

(a) lysing by homogenization the host cells which express the recombinant chondroitinase I enzyme to release the enzyme into the supernatant;

(b) subjecting the supernatant to diafiltration to remove salts and other small molecules;

(c) passing the supernatant through an anion exchange resin-containing column;

(d) loading the eluate from step (c) to a cation exchange resin-containing column so that the enzyme in the eluate binds to the cation exchange column;

(e) obtaining by affinity elution the enzyme bound to the cation exchange column with a solution of chondroitin sulfate, such that the enzyme is co-eluted with the chondroitin sulfate;

(f) loading the eluate from step (e) to an anion exchange resin-containing column and eluting the enzyme with a solvent such that the chondroitin sulfate binds to the column; and

(g) concentrating the eluate from step (f) and crystallizing out the enzyme from the supernatant which contains an approximately 37 kD contaminant.

In the second method, prior to step (b) of the first method just described, the following two steps are performed:

(1) treating the supernatant with an acidic solution to precipitate out the enzyme;

and

(2) recovering the pellet and then dissolving it in an alkali solution to again place the enzyme in a basic environment.

Use of the methods of this invention results in significantly higher yields and purity of each recombinant enzyme than those obtained by adapting for each recombinant enzyme the method previously used for isolating and purifying the native chondroitinase I enzyme from P. vulgaris.

Brief Description of the Figures

Figure 1 depicts a preliminary restriction map for the subcloned approximately 10 kilobase Nsi fragment in pIBI24. The Nsi fragment contains the complete gene encoding chondroitinase I and a portion of the gene encoding chondroitinase II. The restriction sites are shown in their approximate positions. The restriction sites are useful in the constructions described below; other restriction sites present are not shown in this Figure; some are set forth in Example 13 below.

Figure 2 depicts the elution of the recombinant chondroitinase I enzyme from a cation exchange chromatography column using a sodium chloride gradient. The method used to purify the native enzyme is used here to attempt to purify the recombinant enzyme. The initial fractions at the left do not bind to the column. They contain the majority of the chondroitinase I enzyme activity. The fractions at right containing the enzyme are marked "eluted activity". The gradient is from 0.0 to 250 mM NaCl.

Figure 3 depicts the elution of the

recombinant chondroitinase I enzyme from a cation exchange column, after first passing the supernatant through an anion exchange column, in accordance with a method of this invention. The initial fractions at the left do not bind to the column, and contain only traces of chondroitinase I activity. The fractions at right containing the enzyme are marked "eluted activity". The gradient is from 0.0 to 250 mM NaCl.

Figure 4 depicts sodium dodecyl sulfate-polyacrylamide gel chromatography (SDS-PAGE) of the recombinant chondroitinase I enzyme before and after the purification methods of this invention are used. In the SDS-PAGE gel photograph, Lane 1 is the enzyme purified using the method of the first embodiment of the invention; Lane 2 is the enzyme purified using the method of the second embodiment of the invention; Lane 3 represents the supernatant from the host cell prior to purification -- many other proteins are present; Lane 4 represents the following molecular weight standards: 14.4 kD - lysozyme; 21.5 kD - trypsin inhibitor; 31 kD - carbonic anhydrase; 42.7 kD - ovalbumin; 66.2 kD - bovine serum albumin; 97.4 kD - phosphorylase B; 116 kD - beta-galactosidase; 200 kD - myosin. A single sharp band is seen in Lanes 1 and 2.

Figure 5 depicts SDS-PAGE chromatography of the recombinant chondroitinase II enzyme during various stages of purification using a method of this invention. In the SDS-PAGE gel photograph, Lane 1 is the crude supernatant after diafiltration; Lane 2 the eluate after passage of the supernatant through an anion exchange resin-containing column; Lane 3 is the enzyme after elution through a cation exchange resin-containing column; Lane 4 is the enzyme after elution through a second anion exchange resin-containing

column; Lane 5 represents the same molecular weight standards as described for Figure 4, plus 6.5 kD - aprotinin; Lane 6 is the same as Lane 4, except it is overloaded to show the approximately 37 kD contaminant; Lane 7 is the 37 kD contaminant in the supernatant after crystallization of the chondroitinase II enzyme; Lane 8 is first wash of the crystals; Lane 9 is the second wash of the crystals; Lane 10 is the enzyme in the washed crystals after redissolving in water.

Detailed Description of the Invention

Preliminary experiments indicated that E. coli could not use the hydrolysis products yielded by chondroitinase I as a sole carbon source, suggesting that this gene could not be cloned by selecting for its expression in E. coli. Another approach, followed in this application, is to use a physical method to identify DNA fragments that encode the chondroitinase I enzyme. This is accomplished using an appropriately labeled probe for hybridization with individual clones that, together, make up a gene bank comprising the complete genome of P. vulgaris. The probe itself is generated using Polymerase Chain Reaction (PCR) (5). In this procedure, the genomic DNA of P. vulgaris is denatured and oligonucleotides (designed to bracket part of the chondroitinase I gene) are annealed and DNA synthesis is carried out in vitro. This cycle of denaturation, annealing and DNA synthesis using the oligonucleotides as primers is repeated many times (e.g., 30), with the yield of the desired product (the DNA fragment that lies between the two oligonucleotides) increasing exponentially with each cycle.

A putative nucleotide sequence of the

appropriate oligonucleotides is constructed from available amino acid sequence information derived from the protein purified from P. vulgaris bacteria. Once this is done, the DNA fragment produced by PCR is cloned and its DNA sequence determined to verify that it is part of the chondroitinase I gene. It is then labeled and used as a probe to indicate which members of the gene bank actually contain the chondroitinase I gene. Subsequent restriction mapping and Southern hybridization narrows the location to a piece of DNA of approximately four thousand base-pairs (bp). This is then sequenced using the Sanger dideoxy chain termination method (6) to reveal the exact position of the gene and guide the subsequent manipulations used to place the gene into a high-level expression system in E. coli. A fermentation at a 10 liter scale carried out with this E. coli strain containing a recombinant plasmid expressing the P. vulgaris chondroitinase I gene yields a maximum chondroitinase I titer of approximately 600 units/ml (which is the same as 1.2 mg/ml). This yield far exceeds that of the native P. vulgaris fermentation process which had not achieved a titer of more than 2 units/ml.

The process of cloning and expression of the chondroitinase I gene is summarized by the following series of stages:

1) The isolation of P. vulgaris genomic DNA and the construction of a cosmid gene bank.

2) PCR experimentation designed to yield an authentic piece of the chondroitinase I gene for use as a hybridization probe.

3) Colony hybridization studies to identify at least a portion of the chondroitinase I gene.

4) Restriction mapping, Southern hybridi-

zation, DNA sequencing, and chondroitinase I enzyme assays that, collectively, serve to place the location of the chondroitinase I gene more precisely within the cloned DNA.

5) DNA sequence analysis to reveal the exact coding region and location of the chondroitinase I gene.

6) Site-specific mutagenesis, related manipulations, and genetic engineering leading to the regulated, high-level expression of the P. vulgaris gene in E. coli.

These six stages are described in specific detail in Examples 1-7 below. The rationale for the stages is as follows. In the first stage, genomic DNA is obtained. DNA is separated from protein and other material contained in a P. vulgaris fermentation. Study of the genomic DNA is facilitated by the insertion of fragments of the DNA into cosmid vectors. The genomic DNA is digested with an appropriate restriction endonuclease, such as Sau3A, and then ligated into a cosmid vector. The packaged recombinant cosmids containing the P. vulgaris DNA fragments are introduced into an appropriate bacterial host strain, such as an E. coli strain, and the resulting culture is grown to allow gene expression. The gene banks are engineered to contain a marker, such as ampicillin or kanamycin resistance, to assist in the screening of the gene banks for the presence of the chondroitinase I gene.

Applicants have conducted some amino acid sequencing of the native chondroitinase I enzyme. Samples of the enzyme are generated by fermentation of P. vulgaris. Samples may also be obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. The amino acid sequence information is used to design

oligonucleotides for use in screening for the chondroitinase I gene.

In the second stage, oligonucleotides are designed for use in PCR. A first set of oligonucleotides is designed so as to encode a heptapeptide that has minimal degeneracy of its genetic code. Seven amino acids near the amino terminus of the chondroitinase I enzyme (SEQ ID NO:2, amino acids 19-25) are potentially encoded by 512 different nucleotide sequences (SEQ ID NO:6; see Example 2). The number of potential sequences is reduced to 32 by selecting specific nucleotides at the 5′ end, because of the observation that mismatched nucleotides in PCR primers are of less consequence at the 5′ end than at the 3′ end of the primer (7). The sequences of the pool of 32 primers are set out at SEQ ID NOS:7-14.

Applicants have discovered that the approximately 110 kD chondroitinase I enzyme is cleaved proteolytically into an 18,000 MW ("18 kD") fragment and an approximately 90,000 MW ("90 kD") fragment. Furthermore, the 18 kD fragment is further fragmented by treatment with cyanogen bromide and trypsin. The various fragments are then used to design additional sets of oligonucleotide primers for PCR.

Seven amino acids within the 18 kD fragment (SEQ ID NO:2, amino acids 114-120) are potentially encoded by 512 different nucleotide sequences (SEQ ID NO:15; see Example 2). The complementary strand has the same number of potential sequences (SEQ ID NO:16; see Example 2). Using the criteria described above for the first set of oligonucleotides, the number of potential sequences is reduced to 128, whose sequences are set out at SEQ ID NOS:17-24.

Six amino acids located near the amino-terminus of the "90 kD" fragment (SEQ ID NO:2, amino acids 165-170) are potentially encoded by a large number of different nucleotide sequences (SEQ ID NOS:25 and 26; see Example 2). The complementary strand has the same number of potential sequences (SEQ ID NOS:27 and 28; see Example 2). Using the criteria described above for the first set of oligonucleotides, the number of potential sequences is reduced to the sequences set out at SEQ ID NOS:29-36.

PCR amplifications are conducted using these 24 mixtures of oligonucleotides. The most effective amplifications are observed as discrete bands on electrophoretic gels. Products approximately 500 and 350 base pairs (bp) in size are obtained. The approximately 350 bp product is a subfragment of the approximately 500 bp product. The approximately 500 bp product is isolated and, following successive cloning procedures described in Example 2, is isolated as a 455 bp PCR product.

This 455 bp fragment is sequenced and translated into an amino acid sequence which is in virtual agreement with the sequence available from the native chondroitinase I enzyme. The sequences differ by one amino acid; subsequent experiments reveal that the nucleotide and amino acid sequences of the 455 bp fragment are correct, while the native amino acid sequence identification is in error.

In the third stage, the PCR amplification fragment is used as a probe to identify the cosmid gene banks prepared in the first stage which contain the chondroitinase I gene. The PCR fragment is denatured and labelled with, for example, digoxigenin-labelled dUTP (Boehringer-Mannheim, Indianapolis, IN). The cosmid gene banks are then used to infect a

bacterial strain. The resulting colonies are lysed and their DNA subjected to colony hybridization with the labelled probe, followed by exposure to an alkaline phosphatase-conjugated antibody to the digoxigenin-labelled material. Positive clones are visualized and then picked to be grown in selective media.

In the fourth stage, Southern hybridization (8) and restriction mapping are used to localize the position of the chondroitinase I gene within individual clones. The PCR-generated fragment described above is used as a Southern hybridization probe against P. vulgaris genomic DNA that is first digested by restriction enzymes and fractionated. In a second PCR amplification, several of the oligonucleotides described above are used as primers. The results indicate that the portion of the chondroitinase I gene that hybridizes to the probe is carried on several large DNA fragments.

These large DNA fragments are digested to yield individual fragments which are isolated, tested for the presence of chondroitinase I sequences by Southern hybridization, and then subcloned into appropriate vectors. Example 3 details the cloning strategy used. Restriction maps are generated to assist in the identification of the portions of the fragments carrying the desired sequences. In addition, in vitro chondroitinase I assays in which the activity of the enzyme based on measuring the release of unsaturated disaccharide from chondroitin sulfate C at 232 nm are conducted on several samples to assist in the placement and orientation of the chondroitinase I gene. The results of these procedures suggest that a 4.2 kb EcoRV-EcoRI fragment of a larger 10 kb NsiI fragment could contain the

entire chondroitinase I gene.

In the fifth stage, the above-mentioned 4.2 kb fragment is subjected to DNA sequence analysis. The resulting DNA sequence is 3980 nucleotides in length (SEQ ID NO:1). Translation of the DNA sequence into the putative amino acid sequence reveals a continuous open reading frame (SEQ ID NO:1, nucleotides 119-3181) encoding 1021 amino acids (SEQ ID NO:2).

In turn, analysis of the amino acid sequence reveals a 24 residue signal sequence (SEQ ID NO:2, amino acids 1-24), followed by a 997 residue mature (processed) chondroitinase I enzyme (SEQ ID NO:2, amino acids 25-1021).

Signal sequences are required for a complex series of post-translational processing steps which result in secretion of a protein from a host cell. The signal sequence constitutes the amino-terminal end of the protein to be secreted. In most cases, the signal sequence is cleaved off by a specific protease, called a signal peptidase.

The "18 kD" and "90 kD" fragments are found to be adjacent to each other, with the "18 kD" fragment constituting the first 157 amino acids of the mature protein (SEQ ID NO:2, amino acids 25-181), and the "90 kD" fragment constituting the remaining 840 amino acids of the mature protein (SEQ ID NO:2, amino acids 182-1021).

The chondroitinase I enzyme of this invention is expressed using established recombinant DNA methods. Suitable host organisms include bacteria, viruses, yeast, insect or mammalian cell lines, as well as other conventional organisms. The host cell is transformed with a plasmid containing a purified isolated DNA fragment encoding for

chondroitinase I enzyme. The host cell is then cultured under conditions which permit expression of the enzyme by the host cell.

In the sixth stage, the gene is subjected to site-directed mutagenesis to introduce unique restriction sites. These permit the gene to be moved, in the correct reading frame, into an expression system which results in expression of chondroitinase I enzyme at high levels. Such an appropriate host cell is the bacterium E. coli.

As detailed in Example 6 below, two different constructs are prepared. In the first, the three nucleotides immediately upstream of the initiation codon are changed (SEQ ID NO:3, nucleotides 116-118) through the use of a mutagenic oligonucleotide (SEQ ID NO:37). The coding region and amino acid sequence encoded by the resulting construct are not changed, and the signal sequence is preserved (SEQ ID NO:3, nucleotides 119-3181; SEQ ID NO:2).

In a preferred embodiment of this invention, the second construct is used. In the second construct, the site-directed mutagenesis is carried out at the junction of the signal sequence and the start of the mature protein. A mutagenic oligonucleotide (SEQ ID NO:38) is used which differs at six nucleotides from those of the native sequence (SEQ ID NO:1, nucleotides 185-190). The sequence differences result in (a) the deletion of the signal sequence, and (b) the addition of a methionine residue at the amino-terminus, resulting in a 998 amino acid protein (SEQ ID NO:4, nucleotides 188-3181; SEQ ID NO:5).

In the absence of a signal sequence, the enzyme is not secreted. Fortunately, it is not retained within the cell in the form of insoluble

inclusion bodies. Instead, at least some of the enzyme is produced intracellularly as a soluble active enzyme. The enzyme is extracted by homogenization, which serves to lyse the cells and thereby release the enzyme into the supernatant. Even with the signal sequence present, much of the enzyme is not secreted, because it is thought that this expression system provides such high yields of enzyme that it exceeds the capacity of the host cell to secrete that much enzyme.

As described in Example 7 below, the gene lacking the signal sequence is inserted into an appropriate expression vector. One such vector is pET-9A (9; Novagen, Madison, WI), which is derived from elements of the E. coli bacteriophage T7. The resulting recombinant plasmid is designated pTM49-6. The plasmid is then used to transform an appropriate expression host cell, such as the E. coli B strain BL21/(DE3)/pLysS (10; Novagen).

Samples of this E. coli B strain BL21(DE3)/pLysS carrying the recombinant plasmid pTM49-6 were deposited by Applicants on February 4, 1993, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., and have been assigned ATCC accession number 69234.

Expression of the chondroitinase I enzyme using the deposited host cell yields approximately 300 times the amount of the enzyme as was possible using a same size fermentation vessel with native (non-recombinant) P. vulgaris.

After expression of the chondroitinase I enzyme, the supernatant from the host cells is treated to isolate and purify the enzyme. Initial attempts to isolate and purify the recombinant chondroitinase I enzyme do not result in high yields of purified

protein. The previous method for isolating and purifying native chondroitinase I from fermentation cultures of P. vulgaris is found to be inappropriate for the recombinant material.

The native enzyme is produced by fermentation of a culture of P. vulgaris. The bacterial cells are first recovered from the medium and resuspended in buffer. The cell suspension is then homogenized to lyse the bacterial cells. Then a charged particulate such as Bioacryl (Toso Haas, Philadelphia, PA), is added to remove DNA, aggregates and debris from the homogenization step. Next, the solution is brought to 40% saturation of ammonium sulfate to precipitate out undesired proteins. The chondroitinase I remains in solution.

The solution is then filtered and the retentate is washed to recover most of the enzyme. The filtrate is concentrated and subjected to diafiltration with a phosphate to remove the salt.

The filtrate containing the chondroitinase I is subjected to cation exchange chromatography using a cellulose sulfate column. At pH 7.2, 20 mM sodium phosphate, more than 98% of the chondroitinase I binds to the column. The native chondroitinase I is then eluted from the column using a sodium chloride gradient.

The eluted enzyme is then subjected to additional chromatography steps, such as anion exchange and hydrophobic interaction column chromatography. As a result of all of these procedures, chondroitinase I is obtained at a purity of 90-97%. The level of purity is measured by first performing SDS-PAGE. The proteins are stained using Coomassie blue, destained, and the lane on the gel is scanned using a laser beam of wavelength 600 nm. The

purity is expressed as the percentage of the total absorbance accounted for by that band.

However, the yield of the native protein is only 25-35%. The yield is measured as the remaining activity in the final purified product, expressed as a percentage of the activity at the start (which is taken as 100%). In turn, the activity of the enzyme is based on measuring the release of unsaturated disaccharide from chondroitin sulfate C at 232 nm.

This purification method also results in the extensive cleavage of the approximately 110,000 dalton (110 kD) chondroitinase I protein into a 90 kD and an 18 kD fragment. Nonetheless, the two fragments remain non-covalently bound and exhibit chondroitinase I activity.

When this procedure is repeated with homogenate from lysed host cells carrying a recombinant plasmid encoding chondroitinase I, significantly poorer results are obtained. Less than 10% of the chondroitinase I binds to the cation exchange column at standard stringent conditions of pH 7.2, 20 mM sodium phosphate.

Under less stringent binding conditions of pH 6.8 and 5 mM phosphate, an improvement of binding with one batch of material to 60-90% is observed. However, elution of the recombinant protein with the NaCl gradient gives a broad activity peak, rather than a sharp peak (see Figure 2). This indicates the product is heterogeneous. Furthermore, in subsequent fermentation batches, the recombinant enzyme binds poorly (1-40%), even using the less stringent binding conditions. Most of these batches are not processed to the end, as there is poor binding. Therefore, their overall recovery is not quantified.

Based on these results, it is concluded that

the recombinant chondroitinase I enzyme has a reduced basicity compared to the native enzyme, and that the basicity also varies between batches, as well as within the same batch.

It is evident that the method used to isolate and purify the native enzyme is not appropriate for the recombinant enzyme. The method produces low yields of protein at high cost. Furthermore, for large batches, large amounts of solvent waste are produced containing large amounts of a nitrogen-containing compound (ammonium sulfate). This is undesirable from an environmental point of view.

A hypothesis is then developed to explain these poor results and to provide a basis for developing improved isolation and purification methods. It is known that the native chondroitinase I enzyme is basic at neutral pH. It is therefore assumed that the surface of the enzyme has a net excess of positive charges.

Without being bound by this hypothesis, it is believed that, in recombinant expression of the enzyme, the host cell contains or produces small, negatively charged molecules. These negatively charged molecules bind to the enzyme, thereby reducing the number of positive charges on the enzyme. If these negatively charged molecules bind with high enough affinity to copurify with the enzyme, they can cause an alteration of the behavior of the enzyme on the ion exchange column.

Support for this hypothesis is provided by the data described below. In general, cation exchange resins bind to proteins better at lower pH's than higher pH's. Thus, a protein which is not very basic, and hence does not bind at a high pH, can be made to

bind to the cation exchanger by carrying out the operation at a lower pH. At pH 7.2, the native enzyme binds completely to a cation exchange resin. However, the recombinant-derived enzyme, due to the lowered basicity as a result of binding of the negatively charged molecules, does not bind very well (less than 10%). This enzyme can be made to bind up to 70% by using a pH of 6.8 and a lower phosphate concentration (5 mM rather than 20 mM), but heterogeneity and low yield remain great problems. Indeed, only one fermentation results in a 70% binding level; typically, it is much less (less than 10%) even at pH 6.8. This level of binding varies dramatically between different fermentation batches.

This hypothesis and a possible solution to the problem are then tested. If negatively charged molecules are attaching non-covalently to chondroitinase I, thus decreasing its basicity, it should be possible to remove these undesired molecules by using a strong, high capacity anion exchange resin. Removal of the negatively charged molecules should then restore the basicity of the enzyme. The enzyme could then be bound to a cation exchange resin and eluted therefrom in pure form at higher yields.

Experiments demonstrate that this approach indeed provides a solution to the problem encountered with the isolation and purification of the recombinantly expressed chondroitinase I enzyme.

As is discussed below, chondroitinase I is recombinantly expressed in two forms. The enzyme is expressed with a signal peptide, which is then cleaved to produce the mature enzyme. The enzyme is also expressed without a signal peptide, to produce directly the mature enzyme. The two embodiments of this invention which will now be discussed are

suitable for use in purifying either of these forms of the enzyme.

In the first embodiment of this aspect of the invention, the host cells which express the recombinant chondroitinase I enzyme are lysed by homogenization to release the enzyme into the supernatant. The supernatant is then subjected to diafiltration to remove salts and other small molecules. However, this step only removes the free, but not the bound form of the negatively charged molecules. The bound form of these charged species is next removed by passing the supernatant through a strong, high capacity anion exchange resin-containing column. An example of such a resin is the Macro-Prep™ High Q resin (Bio-Rad, Melville, N.Y.). Other strong, high capacity anion exchange columns are also suitable. Weak anion exchangers containing a diethylaminoethyl (DEAE) ligand also are suitable, although they are not as effective. Similarly, low capacity resins are also suitable, although they too are not as effective. The negatively charged molecules bind to the column, while the enzyme passes through the column. It is also found that some unrelated, undesirable proteins also bind to the column.

Next, the eluate from the anion exchange column is directly loaded to a cation exchange resin-containing column. Examples of such resins are the S-Sepharose™ (Pharmacia, Piscataway, N.J.) and the Macro-Prep™ High S (Bio-Rad). Each of these two resin-containing columns has SO_3^- ligands bound thereto in order to facilitate the exchange of cations. Other cation exchange columns are also suitable. The enzyme binds to the column and is then eluted with a solvent capable of releasing the enzyme from the column.

Any salt which increases the conductivity of the solution is suitable for elution. Examples of such salts include sodium salts, as well as potassium salts and ammonium salts. An aqueous sodium chloride solution of appropriate concentration is suitable. A gradient, such as 0 to 250 mM sodium chloride is acceptable, as is a step elution using 200 mM sodium chloride.

A sharp peak is seen in the sodium chloride gradient elution (Figure 3). The improvement in enzyme yield over the prior method is striking. The recombinant chondroitinase I enzyme is recovered at a purity of 99% at a yield of 80-90%.

The purity of the protein is measured by scanning the bands in SDS-PAGE gels. A 4-20% gradient of acrylamide is used in the development of the gels. The band(s) in each lane of the gel is scanned using the procedure described above.

These improvements are related directly to the increase in binding of the enzyme to the cation exchange column which results from first using the anion exchange column. In comparative experiments, when only the cation exchange column is used, only 1% of the enzyme binds to the column. However, when the anion exchange column is used first, over 95% of the enzyme binds to the column.

The high purity and yield obtained with the first embodiment of this invention make it more feasible to manufacture the recombinant chondroitinase I enzyme on a large scale.

In a second embodiment of this aspect of the invention, two additional steps are inserted in the method before the diafiltration step of the first embodiment. The supernatant is treated with an acidic solution to precipitate out the desired enzyme. The

pellet is recovered and then dissolved in an alkali solution to again place the enzyme in a basic environment. The solution is then subjected to the diafiltration and subsequent steps of the first embodiment of this invention.

In comparative experiments with the second embodiment of this invention, when only the cation exchange column is used, only 5% of the enzyme binds to the column. However, when the anion exchange column is used first, essentially 100% of the enzyme binds to the column. The second embodiment provides comparable enzyme purity and yield to the first embodiment of the invention.

Acid precipitation removes proteins that remain soluble; however, these proteins are removed anyway by the cation and anion exchange steps that follow (although smaller columns may be used). An advantage of the acid precipitation step is that the sample volume is decreased to about 20% of the original volume after dissolution, and hence can be handled more easily on a large scale. However, the additional acid precipitation and alkali dissolution steps of the second embodiment mean that the second embodiment is more time consuming than the first embodiment. On a manufacturing scale, the marginal improvements in purity and yield provided by the second embodiment may be outweighed by the simpler procedure of the first embodiment, which still provides highly pure chondroitinase I enzyme at high yields. An additional benefit of the two embodiments of the invention is that cleavage of the enzyme into 90 kD and 18 kD fragments is avoided.

The high purity of the enzyme produced by the two embodiments of this invention is depicted in Figure 4. A single sharp band is seen in the SDS-PAGE

gel photograph: Lane 1 is the enzyme using the method of the first embodiment; Lane 2 is the enzyme using the method of the second embodiment (Lane 3 represents the supernatant from the host cell prior to purification -- many other proteins are present; Lane 4 represents molecular weight standards).

The material deposited with the ATCC can also be used in conjunction with the sequences disclosed herein to regenerate the native chondroitinase I gene sequence (SEQ ID NO:1) or the modified chondroitinase I gene sequence which includes the signal sequence (SEQ ID NO:3) using conventional genetic engineering technology.

Production of native chondroitinase I enzyme in P. vulgaris after induction with chondroitin sulfate does not provide a high yield of enzyme; the enzyme represents approximately 0.1% of total protein present. When the recombinant construct with the signal sequence deleted is used in E. coli, approximately 15% of the total protein is the chondroitinase I enzyme.

In addition to the three DNA sequences just described for the chondroitinase I gene (SEQ ID NOS:1, 3 and 4), the present invention further comprises DNA sequences which, by virtue of the redundancy of the genetic code, are biologically equivalent to the sequences which encode for the enzyme, that is, these other DNA sequences are characterized by nucleotide sequences which differ from those set forth herein, but which encode an enzyme having the same amino acid sequences as those encoded by the DNA sequences set forth herein.

In particular, the invention contemplates those DNA sequences which are sufficiently duplicative of the sequences of SEQ ID NOS:1, 3 or 4 so as to

permit hybridization therewith under standard high stringency Southern hybridization conditions, such as those described in Sambrook et al. (11), as well as the biologically active enzymes produced thereby.

This invention also comprises DNA sequences which encode amino acid sequences which differ from those of the chondroitinase I enzyme, but which are the biological equivalent to those described for the enzyme (SEQ ID NOS:2 and 5). Such amino acid sequences may be said to be biologically equivalent to those of the enzyme if their sequences differ only by minor deletions from or conservative substitutions to the enzyme sequence, such that the tertiary configurations of the sequences are essentially unchanged from those of the enzyme.

For example, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, as well as changes based on similarities of residues in their hydropathic index, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal or C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Therefore, where the terms "chondroitinase I gene" or "chondroitinase I enzyme" are used in

either the specification or the claims, each will be understood to encompass all such modifications and variations which result in the production of a biologically equivalent protein.

The starting point for the cloning and expression of the chondroitinase II gene is partial amino acid sequencing of the mature native chondroitinase II protein obtained from P. vulgaris. The N-terminal sequence of the mature native chondroitinase II protein is found to include the following 22 amino acids:

Leu-Pro-Thr-Leu-Ser-His-Glu-Ala-Phe-Gly-Asp-Ile-Tyr-Leu-Phe-Glu-Gly-Glu-Leu-Pro-Asn-Thr (SEQ ID NO: 40, amino acids 1-22)

The nucleotide sequence determined above for the region encoding the chondroitinase I gene includes an additional approximately 800 base pairs beyond the translation termination codon (SEQ ID NOS:1 and 39, nucleotides 3185-3980). An inspection of this region reveals that the sequence between nucleotides 3307 and 3372 (SEQ ID NOS:1 and 39) encodes the identical 22 amino acids in the same order as the first 22 amino acids of native chondroitinase II.

Furthermore, an ATG initiation codon (SEQ ID NOS:1 and 39, nucleotides 3238-3240) is found upstream of this region and in-frame, indicating that this gene is expressed with a 23 amino acid signal peptide sequence for the export of chondroitinase II (SEQ ID NO:40, amino acids 1-23). Although a Shine-Dalgarno sequence (AGGA; SEQ ID NOS:1 and 39, nucleotides 3225-3228) is found upstream of the initiation codon, there is no apparent promoter sequence, suggesting that both the 110 kD and 112 kD forms of the P. vulgaris

chondroitinase enzyme are expressed as part of a single messenger RNA.

The coding sequence that starts with this ATG was originally not found to be continuous in SEQ ID NO:1, since a termination codon (TAA) was thought to be present in-frame at base-pairs identified as 3607-3609. Re-examination of the sequencing data, however, revealed that a residue was overlooked and that a T should be inserted between nucleotides originally identified as 3593 and 3594. This change restores the open reading frame which then extends through the end of SEQ ID NO: 39 (SEQ ID NOS:1 and 39 include the inserted T as nucleotide 3594). (Thus, the three bases TAA at base-pairs 3608-3610, properly numbered, do not constitute a termination codon.)

With this information available, the cloning and expression of the P. vulgaris chondroitinase II gene is performed in three stages. In the first stage, because the N-terminal sequences are known, a site-specific mutagenesis is carried out. This is necessary in order for this gene to be placed, eventually, directly into the desired T7-based expression vector pET9A that is used (as described above) for the chondroitinase I gene. The mutagenized bases are upstream of the coding region (an AT sequence (SEQ ID NOS:1 and 39, base pairs 3235 and 3236) is replaced by a CA sequence).

The second stage, which can be carried out in parallel with the first, involves the identification, isolation and DNA sequencing of an appropriate DNA fragment which will include the C-terminal coding region of the chondroitinase II gene. The available DNA sequence information is adequate to account for approximately 220 amino acids of an estimated 1000 for the entire chondroitinase II

protein. The missing coding sequences, therefore, would extend for another 2400 base pairs beyond the end of SEQ ID NO: 1.

The third stage involves the assembly of an intact gene for chondroitinase II that has been modified to include the initiation codon as part of an NdeI site and to be followed by a BamHI site downstream of the coding region. This allows a directed insertion of this gene into the pET9A expression vector (Novagen, Madison, WI) without further modification.

Sequencing of the entire assembled gene confirms the presence of the initiation codon at nucleotides 3238-3240, where this codon represents the start of the region coding for the signal peptides at nucleotides 3238-3306, the region coding for the mature protein at nucleotides 3307-6276, and a termination codon at nucleotides 6277-6279 (SEQ ID NO:39). The translation of this sequence results in 1013 amino acids, of which the first 23 amino acids are the signal peptide and 990 amino acids constitute the mature chondroitinase II protein at residues numbered 24-1013 (SEQ ID NO:40). In this construction, the signal peptide is retained, such that the expressed gene is processed and secreted to yield the mature native enzyme structure that has a leucine residue at the N-terminus.

As described in Example 13 below, the gene encoding the chondroitinase II protein is inserted into pET9A and the resulting recombinant plasmid is designated LP^{2}1359. The plasmid is then used to transform an appropriate expression host cell, such as the E. coli B strain BL21(DE3)/pLysS (which is also used for the expression of the chondroitinase I gene.

Samples of this E. coli B strain designated

TD112, which is BL21(DE3)/pLysS carrying the recombinant plasmid LP²1359, were deposited by Applicants on April 6, 1994, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., and have been assigned ATCC accession number 69598.

Expression of the chondroitinase II enzyme using the deposited host cell yields approximately 25 times the amount of the enzyme as was possible using a same size fermentation vessel with native (non-recombinant) P. vulgaris.

After expression of the enzyme, the supernatant from the host cells is treated to isolate and purify the enzyme. Because of the virtually identical isoelectric points and similar molecular weights for the two proteins, the first method described above for isolating and purifying the recombinant chondroitinase I protein is adapted for isolating and purifying the recombinant chondroitinase II protein, and then modified as will now be described.

The need for the modification of the method is based on the fact that the recombinant chondroitinase II protein is expressed at levels approximately several-fold lower than the recombinant chondroitinase I protein; therefore, a more powerful and selective solution is necessary in order to obtain a final chondroitinase II product of a purity equivalent to that obtained for the chondroitinase I protein.

The first several steps of the method for the chondroitinase II protein are the same as those used to isolate and purify the chondroitinase I protein. Initially, the host cells which express the recombinant chondroitinase II enzyme are lysed by

homogenization to release the enzyme into the supernatant. The supernatant is then subjected to diafiltration to remove salts and other small molecules. However, this step only removes the free, but not the bound form of the negatively charged molecules. The bound form of these charged species is next removed by passing the supernatant through a strong, high capacity anion exchange resin-containing column. An example of such a resin is the Macro-Prep™ High Q resin (Bio-Rad, Melville, N.Y.). Other strong, high capacity anion exchange columns are also suitable. Weak anion exchangers containing a diethylaminoethyl (DEAE) ligand also are suitable, although they are not as effective. Similarly, low capacity resins are also suitable, although they too are not as effective. The negatively charged molecules bind to the column, while the enzyme passes through the column. It is also found that some unrelated, undesirable proteins also bind to the column.

Next, the eluate from the anion exchange column is directly loaded to a cation exchange resin-containing column. Examples of such resins are the S-Sepharose™ (Pharmacia, Piscataway, N.J.) and the Macro-Prep™ High S (Bio-Rad). Each of these two resin-containing columns has SO_3^- ligands bound thereto in order to facilitate the exchange of cations. Other cation exchange columns are also suitable. The enzyme binds to the column, while a significant portion of contaminating proteins elute unbound.

At this point, the method diverges from that used for the chondroitinase I protein. Instead of eluting the protein with a a non-specific salt solution capable of releasing the enzyme from the cation exchange column, a specific elution using a

solution containing chondroitin sulfate is used.

This procedure utilizes the affinity the positively charged chondroitinase II protein has for the negatively charged chondroitin sulfate. The affinity is larger than that accounted for by a simple positive and negative interaction alone. It is an enzyme-substrate interaction, which is similar to other specific biological interactions of high affinity, such as antigen-antibody, ligand-receptor, co-factor-protein and inhibitor/activator-protein. Hence, the chondroitin sulfate is able to elute the enzyme from the negatively charged resin. In contrast, the resin-enzyme interaction is a simple positive and negative interaction.

Although affinity elution chromatography is as easy to practice as ion-exchange chromatography, the elution is specific, unlike salt elution. Thus, it has the advantages of both affinity chromatography (specificity), as well as ion-exchange chromatography (low cost, ease of operation, reusability).

Another advantage is the low conductivity of the eluent (approximately 5% of that of the salt eluent), which allows for further ion-exchange chromatography without a diafiltration/dialysis step, which is required when a salt is used. Note, that this is not a consideration in the method for the chondroitinase I protein, because no further ion-exchange chromatography is needed in order to obtain the purified chondroitinase I protein.

There is another reason for not using the method for purifying recombinant chondroitinase I. Chondroitinase II obtained using the chondroitinase I salt elution purification method has poor stability; there is extensive degradation at 4°C within one week. In contrast, chondroitinase II obtained by affinity

elution is stable. The reason for this difference in stability is not known. It is to be noted that chondroitinase I obtained by salt elution is stable.

The cation exchange column is next washed with a phosphate buffer to elute unbound proteins, followed by washing with borate buffer to elute loosely bound contaminating proteins and to increase the pH of the resin to that required for the optimal elution of the chondroitinase II protein using the substrate, chondroitin sulfate.

Next, a solution of chondroitin sulfate in water, adjusted to pH 9.0, is used to elute the chondroitinase II protein, as a sharp peak (recovery 65%) and at a high purity of approximately 95%. A 1% concentration of chondroitin sulfate is used. A gradient of this solvent is also acceptable.

Because the chondroitin sulfate has an affinity for the chondroitinase II protein which is stronger than its affinity for the resin of the column, the chondroitin sulfate co-elutes with the protein. This ensures that only protein which recognizes chondroitin sulfate is eluted, which is desirable, but also means that an additional process step is necessary to separate the chondroitin sulfate from the chondroitinase II protein.

In this separation step, the eluate is adjusted to a neutral pH and is loaded as is onto an anion exchange resin-containing column, such as the Macro-Prep™ High Q resin. The column is washed with a phosphate buffer. The chondroitin sulfate binds to the column, while the chondroitinase II protein flows through in the unbound pool with greater than 95% recovery. At this point, the protein is pure, except for the presence of a single minor contaminant of approximately 37 kD. The contaminant may be a

breakdown product of the chondroitinase II protein.

This contaminant is effectively removed by a crytallization step. The eluate from the anion exchange column is concentrated and the solution is maintained at a reduced temperature, such as 4°C, for several days to crystallize out the pure chondroitinase II protein. The supernatant contains the 37 kD contaminant. Centrifugation causes the crystals to form a pellet, while the supernatant with the 37 kD contaminant is removed by pipetting. The crystals are then washed with water. The washed crystals are composed of the chondroitinase II protein at a purity of greater than 99%.

In a second embodiment of this aspect of the invention for the chondroitinase II protein, two additional steps are inserted in the method before the diafiltration step of the first embodiment. The supernatant is treated with an acidic solution to precipitate out the desired enzyme. The pellet is recovered and then dissolved in an alkali solution to again place the enzyme in a basic environment. The solution is then subjected to the diafiltration and subsequent steps of the first embodiment of this invention.

Acid precipitation removes proteins that remain soluble; however, these proteins are removed anyway by the cation and anion exchange steps that follow (although smaller columns may be used). An advantage of the acid precipitation step is that the sample volume is decreased compared to the original volume after dissolution, and hence can be handled more easily on a large scale. However, the additional acid precipitation and alkali dissolution steps of the second embodiment mean that the second embodiment is more time consuming than the first embodiment. On a

manufacturing scale, the marginal improvements in purity and yield provided by the second embodiment may be outweighed by the simpler procedure of the first embodiment, which still provides highly pure chondroitinase II enzyme at high yields.

Production of native chondroitinase II enzyme in P. vulgaris after induction with chondroitin sulfate does not provide a high yield of enzyme; the enzyme represents approximately 0.1% of total protein present. When the recombinant construct is used in E. coli, approximately 2.5% of the total protein is the chondroitinase II enzyme.

In addition to the DNA sequence just described for the chondroitinase II gene (SEQ ID NO:39), the present invention further comprises DNA sequences which, by virtue of the redundancy of the genetic code, are biologically equivalent to the sequences which encode for the enzyme, that is, these other DNA sequences are characterized by nucleotide sequences which differ from those set forth herein, but which encode an enzyme having the same amino acid sequences as those encoded by the DNA sequences set forth herein.

In particular, the invention contemplates those DNA sequences which are sufficiently duplicative of the sequence of SEQ ID NO:39 so as to permit hybridization therewith under standard high stringency Southern hybridization conditions, such as those described in Sambrook et al. (11), as well as the biologically active enzymes produced thereby.

This invention also comprises DNA sequences which encode amino acid sequences which differ from those of the chondroitinase II enzyme, but which are the biological equivalent to those described for the enzyme (SEQ ID NO:40). Such amino acid sequences may

be said to be biologically equivalent to those of the enzyme if their sequences differ only by minor deletions from or conservative substitutions to the enzyme sequence, such that the tertiary configurations of the sequences are essentially unchanged from those of the enzyme.

For example, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, as well as changes based on similarities of residues in their hydropathic index, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal or C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Therefore, where the terms "chondroitinase II gene" or "chondroitinase II enzyme" are used in either the specification or the claims, each will be understood to encompass all such modifications and variations which result in the production of a biologically equivalent protein.

If desired, one of ordinary skill in the art can ligate together the two pieces of DNA from the two deposits, for example, at the HindIII site at nucleotide 3326, so as to express both the chondroitinase I and chondroitinase II proteins under

the control of the T7 promoter upstream of the coding sequence for chondroitinase I.

In order that this invention may be better understood, the following examples are set forth. The examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention.

Examples

Standard molecular biology techniques are utilized according to the protocols described in Sambrook et al. (11).

Example 1

Isolation Of P. vulgaris Genomic DNA And Construction Of A Cosmid Bank In E. coli

Two 35 ml aliquots (designated A and B) of a P. vulgaris large-scale (1000 liter) fermentation are obtained and centrifuged. Both pellets are resuspended with 7 ml of 0.05M glucose-0.025M Tris-HCl-0.01M EDTA (pH 8) containing 4 mg/ml of egg-white lysozyme. After 30 minutes of incubation at 37°C, 7 ml of 1% SDS-0.16M EDTA-0.02M NaCl (pH 8) are added to sample "A" and incubation is continued at 37°C for another hour.

After the initial lysozyme treatment, sample "B" is centrifuged and the cell pellet taken up with 7 ml of 0.05M glucose-0.025M Tris-HCl-0.01M EDTA (pH 8) containing 40 μg/ml of DNAase-free RNAase and then 7 ml of 1% SDS-0.16M EDTA-0.02M NaCl (pH 8) are added to this resuspended material. Finally, proteinase K (Boehringer Mannheim, Indianapolis, IN) is added to both samples to a final concentration of 100 μg/ml and

incubation is continued overnight at 37°C.

The next day, the samples are extracted once with an equal volume (14 ml) of equilibrated phenol followed by two further extractions in which the samples are extracted with 7 ml of phenol followed by the addition of 7 ml of chloroform, continued shaking and finally, centrifugation to separate the two phases. The DNA is precipitated by adding one-quarter volume of 5M ammonium acetate and 0.6 volumes of isopropanol followed by centrifugation. The pelleted DNA is rinsed once with 70% (v/v) ethanol, dried under vacuum and then resuspended with 1 ml of TE (0.01M Tris-HCl-0.001M EDTA, pH 7.4). The nucleic acid concentration for sample "A" is 1.2 mg/ml while that for sample "B" is 1.3 mg/ml, as determined by their ultraviolet absorption at 260 nm.

Fragmentation of the genomic DNA to yield pieces of a size suitable for insertion into cosmid vectors (approximately 25-35 kilobases (kb)) is accomplished by partial digestion with the restriction endonuclease Sau3A. Duplicate 0.2 ml reactions are set up (one with preparation "A" and the other with DNA from preparation "B"), each containing 100 μg of the P. vulgaris genomic DNA, 0.1M NaCl, 0.01M $MgCl_2$, 0.01M Tris-HCl (pH 7.5) and 80 units of the enzyme Sau3A.

Incubation is carried out at 37°C and 25 μl aliquots are removed at appropriate time points (5,6,7,8,9,10,11 and 20 minutes) and added to 25 μl of 0.2M EDTA (pH 8). The individual samples are heated to 70°C and then 10 μl are removed for a size-distribution analysis on an agarose gel. The sample obtained after five minutes of Sau3A digestion of preparation "A" and that obtained after 6 minutes with preparation "B" are chosen for further use.

In each case, an aliquot (4 μl, which is approximately equal to 2 μg) of the chosen partial digest is ligated to the appropriate "left" and "right" arms of the cosmid vector DNA using approximately 1 μg and 2 μg of each, respectively, in 10 μl reactions containing 0.066M Tris-HCl (pH 7.4), 0.01M $MgCl_2$, 0.001M ATP, and 400 units (as defined by the manufacturer (New England Biolabs, Beverly, MA)) of T4 DNA ligase. Incubation is carried out at 11°C overnight. The "left" and "right" arms of the cosmids are DNA fragments which, when ligated to an appropriately sized piece of P. vulgaris DNA, comprise a recombinant molecule of approximately 35-50 kb. Both arms contain "cos" sites which are recognized by the packaging enzymes in the next step. In addition, these arms carry the origin of replication and ampicillin-resistance functions of pIBI24 (International Biochemical Inc., New Haven, CT).

Each of the above ligase reactions is added to one tube of a λ packaging extract (Packagene™, a trademark of Promega Corp., Madison, WI) and the reaction is allowed to proceed at room temperature for two hours, at which point 0.5 ml of PDB (0.1M NaCl-0.01M Tris-HCl (pH 7.9)-0.01M $MgSO_4$) is added followed by approximately 0.05 ml of chloroform. Each tube of packaged DNA is, therefore, a gene bank of the P. vulgaris genome.

Because this method of construction creates a pool of infectious particles (i.e., λ phage heads filled with the cosmid vector joined to approximately 25 to 35 kb of P. vulgaris DNA), the number of potential clones is quantitated by adsorbing an aliquot of the packaged material to an appropriate, sensitive E. coli host strain, and then after outgrowth, plating the mixture on selective media.

For example, an overnight culture of the E. coli strain ER1562 (New England Biolabs, Beverly, MA) grown in 20-10-5 medium is diluted 1:20 into fresh media (20-10-5 supplemented with 1% maltose) and grown for three hours at 37°C. The cells (1 ml) are then centrifuged, resuspended with PDB (0.2 ml) and 0.02 ml of the appropriate gene bank added. After adsorption for twenty minutes at 37°C, the samples are diluted to 2 ml with 20-10-5 medium and grown at 37°C for 30 minutes. The culture is then spread on 20-10-5 plates containing 100 μg/ml of ampicillin and colonies scored after overnight incubation at 37°C. The results indicate that there are approximately 68,000 and 95,000 infectious particles (potential cosmid clones) present in the two samples, designated PV1-GB and PV2-GB, corresponding to the "A" and "B" preparation of P. vulgaris genomic DNA, respectively.

In addition, four other P. vulgaris gene banks are prepared, as above, using two different cosmid vectors. These two cosmids differ from the above-mentioned vectors in that a kanamycin resistance determinant is used in one case rather than the ampicillin resistance, while in the other, the replication functions of pBR322 (New England Biolabs, Beverly, MA) are used instead of those of pIBI24. These four "libraries," designated L1974, L1975, L1976, and L1977, contain, respectively, approximately 18,000 (amp^r), 34,000 (amp^r), 13,000 (kan^r) and 15,000 (kan^r) members. Aliquots of each of these six gene banks are screened for the presence of the P. vulgaris chondroitinase I gene (see below).

Example 2

PCR Experimentation Designed To Yield An Authentic Piece Of The Chondroitinase I Gene For Use As A Hybridization Probe

The Polymerase Chain Reaction (PCR) (5) allows the geometric amplification of a DNA sequence that lies between oligonucleotide primers that can be extended by a DNA polymerase in vitro. The enzyme used in these experiments is the Taq DNA polymerase (isolated originally from Thermus aquaticus), which is preferred because of its thermotolerance which allows it to survive the repeated DNA denaturation steps that are carried out at 94°C.

In order for this method to be employed successfully, the oligonucleotides used must have sequences that are as close as possible to those of the target sequence -- the P. vulgaris chondroitinase I gene. An approximation of that sequence can be derived from the limited available amino acid sequence data. To minimize uncertainty in the sequence presented by the degeneracy of the genetic code (a given amino acid can be encoded by up to six codons), the first approximation involves choosing an amino acid sequence that has the least degeneracy. For example, in the amino-terminal sequence of the P. vulgaris chondroitinase I gene, there are the following consecutive amino acids: His-Phe-Ala-Gln-Asn-Asn-Pro (SEQ ID NO:2, amino acids 43-49).

This amino acid sequence could be encoded by any one of 512 different nucleotide sequences, represented as 5'-CAY-TTY-GCN-CAR-AAY-AAY-CCN-3' (SEQ ID NO:6), where R stands for purine (A or G), Y for pyrimidine (C or T), and N indicates that any one of the four nucleotides (A T, G, or C) at this position

will constitute a nucleotide sequence that could encode the indicated amino acid sequence. One possible approach would be to synthesize an oligonucleotide mixture containing a total of 512 different oligonucleotides, represented as:

5'-CA(TC)-TT(TC)-GC(GATC)-CA(GA)-AA(TC)-AA(TC)-CC-(GATC)-3' (SEQ ID NO:6).

Although use of such mixtures in PCR has been successful, another approach is to use a number of oligonucleotide mixtures, each of which is made up of a relatively smaller set of nucleotide sequences. In order to simplify this further, advantage is taken of the observation (7) that mismatched nucleotides in PCR primers are of less consequence at the 5'-end of the primer than they are at the 3'-end. Using these criteria, a set of eight oligonucleotides (each made up of four unique sequences) is designed, where the individual sets of oligonucleotides have the following sequences:

1. 5'-CAC-TTC-GC(GATC)-CAA-AAT-AAT-CC-3' (SEQ ID NO:7)
2. 5'-CAC-TTC-GC(GATC)-CAA-AAC-AAC-CC-3' (SEQ ID NO:8)
3. 5'-CAC-TTC-GC(GATC)-CAA-AAC-AAT-CC-3' (SEQ ID NO:9)
4. 5'-CAC-TTC-GC(GATC)-CAA-AAT-AAC-CC-3' (SEQ ID NO:10)
5. 5'-CAC-TTC-GC(GATC)-CAG-AAT-AAT-CC-3' (SEQ ID NO:11)
6. 5'-CAC-TTC-GC(GATC)-CAG-AAC-AAC-CC-3' (SEQ ID NO:12)
7. 5'-CAC-TTC-GC(GATC)-CAG-AAC-AAT-CC-3' (SEQ ID NO:13)
8. 5'-CAC-TTC-GC(GATC)-CAG-AAT-AAC-CC-3' (SEQ ID NO:14)

One of these pools is perfectly matched for the first eleven nucleotides (counting from the 3-end), and, furthermore, within this pool of four oligonucleotides, one is a perfect match for the first

fourteen nucleotides. This is important because it permits stringent annealing conditions to be used that discriminate against imperfect matches that give rise to PCR products that are unrelated to the chondroitinase I gene.

A further aid in the design of oligonucleotides to be used in these PCR experiments is derived from the observation that the P. vulgaris 110 kD chodroitinase enzyme appears to have a structure that leaves one particular region hypersensitive to proteolytic cleavage. The result of this hydrolysis is that the normally approximately 110 kD protein is split into two predominant species of 18 kD and approximately 90 kD. The amino-terminal sequences of the "110 kD" protein and the "18 kD" fragment are the same, while that for the "90 kD" has been found to be different.

The "18 kD" peptide is further fragmented by treatment with cyanogen bromide and trypsin and the resulting oligopeptides sequenced, affording still more information with which to design oligonucleotides for PCR. This information from the "18 kD" and "90 kD" regions is also valuable because the locations of these amino acid sequences relative to each other and the N-terminal sequences of the intact protein are well defined. In fact, the nucleotide distance between the regions encoding the N-termini of the "110 kD" and "90 kD" entities can be predicted to be approximately 400-500 bp.

Two further sets of oligonucleotide pools are then designed with one further consideration: The first eight oligonucleotides hybridize to one strand of the DNA and, during the in vitro DNA synthesis, they are extended toward the "90 kD" N-terminal coding sequences. Consequently, the oligonucleotides

corresponding to amino acid sequences from within the "18 kD" peptide and at the N-terminus of the "90 kD" peptide must be designed so that they anneal to the complementary DNA strand of the P. vulgaris genome, so that they extend, in vitro, toward the region encoding the N-terminus of the intact protein.

In this way, the oligonucleotides effectively "bracket" the region of the P. vulgaris chromosome that encodes the N-terminal region of the chondroitinase I gene. It is worth noting that the PCR methodology offers an extremely large potential amplification of this bracketed region. Thirty PCR cycles, in theory, increase the number of copies of this DNA segment by a factor of one billion. This allows the use of very small quantities of P. vulgaris genomic DNA as a template which will yield, potentially, microgram amounts of synthesized product which can be readily visualized, isolated and cloned.

Using the above logic, oligonucleotide mixtures are designed based on the following amino acid sequence that is found within the "18 kD" peptide: Glu-Ala-Gln-Ala-Gly-Phe-Lys (SEQ ID NO:2, amino acids 138-144). This heptapeptide is encoded by the following nucleotide sequences:

5'-GAR-GCN-CAR-GCN-GGN-TTY-AAR-3' (SEQ ID NO:15).

The complementary strand, therefore, has the following sequences:

5'-YTT-RAA-NCC-NGC-YTG-NGC-YTC-3' which is the same as 5'-(CT)TT-(AG)AA-(GATC)CC-(GATC)GC-(CT)TG-(GATC)GC-(CT)TC-3' (SEQ ID NO:16).

Using the same criteria as described above

for the first set of eight oligonucleotides, a further set of eight oligonucleotides (each made up of 16 unique sequences) is designed, where the individual sets of oligonucleotides have the following sequences:

9. 5'-TT-GAA-(AG)CC-(GATC)GC-(CT)TG-GGC-TTC-3' (SEQ ID NO:17)

10. 5'-TT-GAA-(AG)CC-(GATC)GC-(CT)TG-AGC-TTC-3' (SEQ ID NO:18)

11. 5'-TT-GAA-(AG)CC-(GATC)GC-(CT)TG-TGC-TTC-3' (SEQ ID NO:19)

12. 5'-TT-GAA-(AG)CC-(GATC)GC-(CT)TG-CGC-TTC-3' (SEQ ID NO:20)

13. 5'-TT-GAA-(AG)CC-(GATC)GC-(CT)TG-GGC-CTC-3' (SEQ ID NO:21)

14. 5'-TT-GAA-(AG)CC-(GATC)GC-(CT)TG-AGC-CTC-3' (SEQ ID NO:22)

15. 5'-TT-GAA-(AG)CC-(GATC)GC-(CT)TG-TGC-CTC-3' (SEQ ID NO:23)

16. 5'-TT-GAA-(AG)CC-(GATC)GC-(CT)TG-CGC-CTC-3' (SEQ ID NO:24)

Unlike oligonucleotides 1-8 above, one base is deleted from the 5' end of oligonucleotides 9-16 in order to reduce the number of sequence permutations.

In this case, one pool has a perfect match for the first eight nucleotides at the 3'-end, while 50% of this same pool has an eleven-nucleotide perfect match with the genomic DNA of P. vulgaris encoding chondroitinase I.

For a third set of oligonucleotide mixtures, the following amino acid sequence, obtained as part of the N-terminal amino acid sequence of the "90 kD" peptide, is used: Gly-Ala-Lys-Val-Asp-Ser (SEQ ID NO:2, amino acids 189-194). This hexapeptide can be

encoded by the following nucleotide sequences:

5'-GGN-GCN-AAR-GTN-GAY-TCN-3' (SEQ ID NO:25)
or
5'-GGN-GCN-AAR-GTN-GAY-AGY-3' (SEQ ID NO:26)

The complement of this sequence is:

5'-NGA-RTC-NAC-YTT-NGC-NCC-3' (SEQ ID NO:27)
or
5'-RCT-RTC-NAC-YTT-NGC-NCC-3' (SEQ ID NO:28)

These possible sequences are represented using the following oligonucleotide mixtures:

17. 5'-GA-GTC-(GATC)AC-(TC)TT-(AG)GC-GCC-3' (SEQ ID NO:29)
18. 5'-GA-GTC-(GATC)AC-(TC)TT-(AG)GC-ACC-3' (SEQ ID NO:30)
19. 5'-GA-GTC-(GATC)AC-(TC)TT-(AG)GC-TCC-3' (SEQ ID NO:31)
20. 5'-GA-GTC-(GATC)AC-(TC)TT-(AG)GC-CCC-3' (SEQ ID NO:32)
21. 5'-GA-GTC-(GATC)AC-(TC)TT-(TC)GC-GCC-3' (SEQ ID NO:33)
22. 5'-GA-GTC-(GATC)AC-(TC)TT-(TC)GC-ACC-3' (SEQ ID NO:34)
23. 5'-GA-GTC-(GATC)AC-(TC)TT-(TC)GC-TCC-3' (SEQ ID NO:35)
24. 5'-GA-GTC-(GATC)AC-(TC)TT-(TC)GC-CCC-3' (SEQ ID NO:36)

Unlike oligonucleotides 1-8 above, one base is deleted from the 5' end of oligonucleotides 17-24 in order to reduce the number of sequence

permutations.

In this case, one oligonucleotide mixture has half of its members perfectly matched for the first eight nucleotides at the 3'-end, and one quarter of the oligonucleotides in the pool are perfectly matched for eleven nucleotides at the 3'-end.

These twenty-four oligonucleotide mixtures are purchased from Biosynthesis, Inc. (Denton, TX), and are provided as fully deprotected, purified and lyophilized samples. In each case (except oligonucleotide #20), 5 O.D. units of synthetic DNA are obtained. This is resuspended in 0.5 ml of water to yield a solution that contains approximately 50-60 pmoles of oligonucleotide per microliter. The remaining sample (oligonucleotide #20) contains 15 O.D. and is resuspended with one ml of water to give a solution with approximately 90 pmole/μl.

A typical 50 μl PCR reaction contains approximately 20 ng of P. vulgaris genomic DNA as template; 200 μM each of dATP, dGTP, dCTP, dTTP; 50mM KCl; 10mM Tris-HCl (pH 8.4); 1.5 mM $MgCl_2$; 0.01% gelatin; 2.5 units of Ampli-Taq™ DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT); and 50 pmoles of each oligonucleotide pool to be tested. The reactions are overlaid with mineral oil (Plough) and incubated in a Perkin-Elmer/Cetus Thermalcycler™.

For each cycle, the instrument is programmed to denature the template DNA at 94°C for 1.25 minutes, anneal the oligonucleotide primers to the denatured template at 60°C or 62°C for one minute, and to extend these primers via DNA synthesis at 72°C for 2.25 minutes. Thirty such cycles are carried out in an experimental amplification. The products are analyzed by running an aliquot on a 4% NuSieve™ (FMC Biochemicals, Rockland, ME) GTG gel containing

approximately 0.5 μg/ml ethidium bromide using either Tris-borate or Tris-acetate buffers at either full or half strength. These gels are usually run overnight at approximately 1V/cm and photographed on a long wavelength UV transilluminator using a red filter and Polaroid Type 57 film.

PCR experiments are run testing the pairwise combinations between oligonucleotide pools #1-8 (derived from the "110 kD" amino-terminal sequence of chondroitinase I), pools #9-16 (derived from a peptide sequence contained within the "18 kD" fragment), and pools #17-24 (derived from the amino-terminal sequence of the "90 kD" fragment). The most effective amplifications observed (based on the visual yield of a discrete DNA band detected on gel electrophoretic analysis of the reaction products) are between oligonucleotide pools #4 and #18, and pools, #4 and #9,10,11, or 12. In general, the other pools, which differ by one nucleotide from these pools, also yield some amplification. A difference of two nucleotides results, essentially, in no observed product. It is important to note, however, that the annealing temperatures are deliberately kept at 60-62°C to enhance such discrimination.

PCR amplifications using oligonucletide pools #4 and #18 yield a product of approximately 500 bp as estimated relative to size standards (pBR322 digested with MSP-1 (New England Biolabs, Beverly, MA) ranging from 30 to 700 bp on NuSieve™ agarose gels. The product from the use of oligonucleotide pool #4 combined with pools #9, 10, 11, or 12 is approximately 350 bp in length. Furthermore, the larger product could be isolated from an agarose gel, diluted a thousand-fold, and then used as the template in a second PCR reaction employing oligonucleotide pools #4

and #9 as primers, which yield a product of approximately 350 bp. That is, the smaller PCR product is synthesized from the larger one in agreement with what would be expected if these sequences were all derived from the P. vulgaris chondroitinase I gene. This indicates that the desired region of the genome is amplified.

The larger PCR product is isolated from an agarose gel using a Qiaex™ extraction procedure according to the manufacturer's instructions (Qiagen, Chatsworth, CA). The isolated DNA is then subjected to a "fill-in" reaction (11) to remove the extra, protruding adenine residue that Taq DNA polymerase tends to add to the 3'-end of DNA in a template-independent reaction (12). The isolated DNA is then treated with T_4 polynucleotide kinase to add a phosphate moiety to the 5'-ends of the PCR products to allow them to be joined to the vector DNA. After these treatments, the PCR product is ligated to pIBI24, a high copy vector containing a polylinker (IBI, New Haven, CT), that is first sequentially digested with PstI, "filled-in" and then treated with calf intestinal alkaline phosphatase (Boehringer-Mannheim).

Once the PCR product is cloned into pIBI24, it is removed as an EcoRI-HindIII fragment by virtue of the restriction sites within the polylinker carried by the plasmid. This fragment is then cloned into both M13mp18 and M13mp19 (13; New England Biolabs, Beverly, MA) after cleavage with both EcoRI and HindIII and then phosphatased. Single stranded DNA corresponding to these constructions is then isolated and subjected to DNA sequence analysis using an Applied Biosystems (Foster City, CA) instrument and Taq sequencing kit. The results indicate that the

larger PCR product is 455 bp in length. As expected, the ends of the fragment are derived from the oligonucleotide pools used as primers.

The DNA sequence is translated into an uninterrupted amino acid sequence that is in agreement (with one exception described below) with the available data obtained by amino acid sequence analysis on the native chondroitinase I protein itself, including, for example, a twelve residue oligopeptide (SEQ ID NO:2, amino acids 133-144). An eight residue oligopeptide derived from the DNA sequence (SEQ ID NO:2, amino acids 71-78) also matches a previously sequenced oligopeptide derived by a combination of trypsin digestion and cyanogen bromide treatment of the native protein. The only discrepancy between the two sequences is at amino acid residue #162 of the mature protein (SEQ ID NO:2, amino acid 186), where the DNA sequence codes for an arginine, while the native protein sequence indicates a leucine.

Since a single nucleotide alteration would change a leucine codon (CTT) to an arginine codon (CGT), an initial interpretation suggests that this may be caused by a lack of perfect incorporation fidelity by the Taq DNA polymerase during the in vitro amplification process. However (see below) later results indicate that the DNA sequence is correct, rather than the amino acid sequence obtained by analyzing the native enzyme. These results also indicate that the "18 kD" and the "90 kD" fragments are, in fact, contiguous pieces of the chondroitinase I protein that has been cleaved (presumably by a contaminating protease), predominately between residues #157 (Gln) and #158 (Asp) of the mature protein (SEQ ID NO:2, between amino acids 181 and 182). All of the above information supports the

interpretation that the cloned DNA (at least that portion that is bracketed by the oligonucleotide primers) generated by PCR amplification represents part of the authentic chondroitinase I gene of P. vulgaris and, therefore, can be used as a probe to identify cosmid clones that carry the intact gene.

Although it is possible to isolate the entire gene coding for a protein of interest using PCR amplification (thereby avoiding construction of a gene bank and many of the other steps described below) by employing oligonucleotide primers derived from the amino-terminus of the protein coupled with primers derived from the carboxyl-terminal amino acid sequence, there are several potential problems in this approach. In the case of the P. vulgaris chondroitinase I, the problems include: (1) the assumption that the protein being sequenced has not been processed at either end (not likely to be true, for example, with a secreted protein), (2) the occasional lack of fidelity exhibited by Taq DNA polymerase during PCR reactions, and (3) the rather large size of the bracketed region of the DNA that is to be amplified which was expected to be approximately 3000 bp (deduced from the apparent molecular weight of approximately 110 kD). Consequently, the approach of constructing a gene bank is selected.

Example 3

Generation Of A Labeled Probe, Colony Hybridization And Identification Of Positive Cosmid Clones From The P. vulgaris Gene Bank

The cloned PCR product corresponding to the 455 bp near the amino-terminal coding portion of the P. vulgaris chondroitinase I gene is released from the

plasmid DNA into which it had been cloned by digestion with the restriction enconuclease SalI. This is a consequence of the presence of one SalI site within the polylinker sequence and a second SalI site within the cloned PCR amplification product (this is fortuitous in that the latter SalI site is derived from the nucleotide sequence of oligonucleotide pool #18 near its 5'-end; in fact, there is no recognition site for SalI within the P. vulgaris chondroitinase I gene itself). A total of approximately 260 μg of plasmid DNA is digested with SalI and the products separated by electrophoresis on a NuSieve™ GTG agarose gel. The desired approximately 450 bp fragment is isolated using a Qiaex™ extraction protocol. The fragment is then denatured by heating at 95-100°C for 5-15 minutes, followed by rapid cooling. The denatured fragment is then labelled with digoxigenin-labelled dUTP (Boehringer-Mannheim, Indianapolis, IN) in two 200 μl reactions.

Aliquots of the six P. vulgaris cosmid gene banks described in Example 1 above are used to infect the E. coli strain ER1562 described above and a total of approximately 10,000 colonies are obtained on the appropriate selective plates. These colonies (on a total of 50 plates) are replica plated onto two nylon membranes on selective agar as well as to a third selective plate. After overnight incubation, the colonies on the filters are lysed by sequentially treating with 10% sodium dodecyl sulfate (SDS) and 0.5 M NaOH for 5-30 minutes each. The cells from the lysed colonies are neutralized by being placed on sheets saturated with 1 M Tris-HCl (pH 7.4) (twice) and then on paper saturated with 2X standard saline citrate prior to vacuum drying at 80°C. The DNA from the lysed colonies is then fixed to the membranes.

The filters are then washed by incubation of the filters at 42°C with agitation for 1-3 hours, using at least 10 ml/filter of 0.05 M Tris HCl, 0.5-1 M NaCl/0.001 M EDTA, pH 8, 0.1% SDS and 0.05 mg/ml proteinase K. The filters are then rinsed with 2 X SSC and pre-hybridized by incubation with a hybridization buffer at 65°C for 1-3 hours. The filters are then hybridized overnight at 65-68°C using the digoxigenin-labeled probe described above (0.5-50 ng/ml in a hybridization solution). The hybridized filters are washed with SSC and SDS, re-blocked with a blocking reagent (Component #11 of DNA Labelling and Detection Kit, Nonradioactive, Boehringer Mannheim, Indianapolis, IN) and exposed to polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase.

The positive clones are visualized by incubation of the antibody-labeled filters in the presence of BCIP (bromo-chloro-indolyl-phosphate) and NBT (nitro-blue tetrazolium). The presence of the desired DNA fragment within a colony will result in a dark brownish-purple spot in the filter after this hybridization procedure. After approximately four hours, the developed filters are used as templates to guide the selection of a total of 117 clones which are then picked to selective media. A small-volume (10 ml) culture ("Miniprep") of each of these clones is grown in selective media and plasmid DNA is then isolated using materials and protocols supplied by Qiagen.

Example 4

Restriction Mapping And Southern Hybridization Used To Localize The Position Of The Chondroitinase I Gene Within Individual Clones

A number of approaches are used to guide the selection of particular cosmid clones for further study. One is to carry out Southern hybridization (8) using the same PCR-generated fragment as a probe against P. vulgaris genomic DNA that had been digested by a number of restriction enzymes and then fractionated on an agarose gel prior to transfer to a nylon membrane. The probe is labeled with digoxigenin-dUTP by including this nucleotide analogue in a PCR amplification. In this reaction, the gel-purified product of a previous PCR amplification (that using P. vulgaris genomic DNA as template) is diluted 10,000-fold and serves as the template in a second PCR amplification.

This latter reaction is made up as a 0.5 ml mixture, which is then divided into ten individual tubes and amplified as described above for 25 cycles using oligonucleotide pools #2 and #10 (see above) as the primers. The normal complement of deoxyribonucleoside triphosphates is replaced with a digoxigenin-dUTP labeling mixture from the manufacturer (Boehringer-Mannheim, Indianapolis, IN), which yields a final concentration of 100 μM each of dATP, dCTP and dGTP, 65 μM dTTP and 35 μl digoxigenin-dUTP. The reactions are pooled and precipitated according to the manufacturer's recommendations. An aliquot of the resuspended product is examined by gel electrophoresis and exhibits a single band between approximately 300 and approximately 400 bp in length as expected for the "smaller" PCR product described

above.

To avoid problems encountered with the highly viscous P. vulgaris genomic DNA preparation, the DNA (approximately 5 μl) is diluted into large (0.35 ml) volumes for digestion with the various restriction enzymes. The DNA is then concentrated by ethanol precipitation prior to fractionation on agarose gels and transfer to nylon membranes. The data obtained in these experiments indicates that the chondroitinase I gene (at least that portion that hybridizes to the N-terminal coding region represented by the probe described above) is carried on a BstYI fragment of approximately 2800 bp, an EcoRV fragment of 5400 bp, and on large (equal to or greater than approximately 10kb) DNA fragments generated by NsiI, BglII, HindIII, and StyI.

Large scale cultures (500 ml) of a number of hybridizing cosmid clones are grown and plasmid DNA is isolated from these cultures for use in mapping the location of the chondroitinase I gene. The DNA of the gene is expected to represent only approximately 10 per cent of the P. vulgaris DNA carried within each cosmid. A number of these clones are digested with BstYI and NsiI and the products are fractionated on an agarose gel. Individual fragments are then isolated, a portion tested for the presence of chondroitinase I sequences by Southern hybridization, and then subcloned into appropriate vectors.

Two of these fragments are of special interest. The first, a BstYI fragment of approximately 2800 bp, is observed in a number of cosmid clones, including those designated #2 and #45. The DNA isolated from these two cosmid clones is designated LP^2 751 and LP^2 760. With LP^2 760, the approximately 2800 bp BstYI fragment is well separated

from the other BstYI fragments and is therefore more readily subcloned into another vector designated pT660-3. The plasmid designated pT660-3 is a derivative of pBR322 in which the DNA from a point immediately downstream of the promoter for tetracycline resistance (approximately bp 80) as far as the PvuII site (approximately bp 2070) is deleted and replaced with a BamHI linker. Similarly, the approximately 10 kb NsiI fragment (which hybridizes with the chondroitinase probe described above) is readily isolated from a digest performed on LP^2 751. These two fragments are referred to as the "2800 bp BstYI" fragment and the "10 kb NsiI" fragment.

The 2800 bp BstYI fragment is small enough to permit a second restriction enzyme digestion on this piece of DNA in order to obtain a fragment suitable for DNA sequence analysis. This is important because the hybridization experiments serve to identify the N-terminal coding region of the chondroitinase I gene, due to how the probe is derived. This procedure does not, however, indicate to which side the rest of the gene is located. Given the relative size of the probe (less than 500 bp) compared to the predicted size of the intact gene (greater than 3000 bp), this is not a trivial consideration. The nucleotide sequence, however, clearly indicates in which direction the gene would be "read" and therefore, which restriction fragments should be cloned in order to obtain the entire gene.

The subcloned 2800 bp BstYI fragment contains two internal EcoRV sites, which suggests that the resulting fragments might be small enough for DNA sequencing. However, the EcoRV sites are symmetrically placed within the 2800 bp BstYI fragment; each EcoRV site is approximately 1200 bp

from one end, with the space between them equal to approximately 400 bp. The subcloned fragment is digested asymmetrically by taking advantage of unique restriction sites present within the vector. In this manner, the "halves" of the 2800 bp BstYI fragment are distinguished physically and, by Southern hybridization, the "end" that contains the chondroitinase I N-terminal coding region is ascertained. Once this is done, the appropriate piece, which is a HindIII-EcoRV fragment of approximately 1200 bp, is subcloned into both M13mp18 and M13mp19 vectors which are first digested with both HindIII and SmaI and subsequently treated with calf intestinal alkaline phosphatase. The DNA sequence derived from these subclones reveals a number of features that clearly establish the location of the chondroitinase I gene, as well as the direction in which it is read.

Starting with nucleotide #183 in this sequence (SEQ ID NO:1, nucleotide 191), a coding region is observed which matches the first thirty previously-identified amino acids of the P. vulgaris chondroitinase I enzyme. Preceding this sequence, it is possible to discern a number of other features by their analogy to corresponding sequence motifs from previously analyzed E. coli genes. These features include: (1) nucleotides 32-37 (SEQ ID NO:1, nucleotides 40-45) which match in three of six positions with the consensus "-35" region of a promoter and, after a 17 nucleotide space, a "-10" region of a promoter (matching in six of seven positions with the consensus "-10" region); (2) a putative "Shine-Dalgarno" sequence can be noted between nucleotides 98-103 (SEQ ID NO:1, nucleotides 106-111); and (3) there is an in-frame ATG initiation codon at nucleotides 111-113 (SEQ ID NO:1, nucleotides

119-121), which indicates that the P. vulgaris chondroitinase I enzyme is synthesized with a 24 amino acid signal sequence which is, presumably, removed as the protein is transported across the inner membrane.

The second fragment that is subcloned (into a pIBI24 derivative that is first modified to include an NsiI restriction site in place of the PstI site normally present in the polylinker of this vector) is the approximately 10 kb NsiI fragment. Digestion of this approximately 14 kb recombinant molecule (the approximately 10 kb NsiI fragment in pIBI24) with EcoRV yields four fragments of approximately 9 kb, 2.3 kb, 2.1 kb, and 0.4 kb. Southern hybridization analysis using the probe derived from the N-terminal amino acid sequence indicates that the related chondroitinase gene sequences are contained within the largest fragment (the approximately 9 kb EcoRV fragment).

Since there is no other fragment larger than 2.9 kb (the size of pIBI24 which has no internal EcoRV recognition sites), this approximately 9 kb EcoRV fragment must contain the vector as well as P. vulgaris DNA. A double digestion of this recombinant molecule with NsiI and EcoRV releases the pIBI24 vector as a 2.9 kb fragment; it also yields fragments of approximately 4.5 kb, 2.3 kb, 2.1 kb, 1.0 kb and 0.4 kb. Taken together (along with the information presented above on the 2.8 kb BstYI fragment which has two internal EcoRV sites separated by approximately 0.4 kb), an initial restriction map is constructed.

A double digestion with EcoRV and HindIII releases fragments of approximately 4.1 kb, 2.3 kb, 2.1 kb, 2.0 kb, 1.3 kb, 1.1 kb and 0.4 kb. Three of these fragments (2.3 kb, 2.1 kb, and 0.4 kb) are apparently EcoRV fragments that have not been cut by

HindIII. Again, the only fragment larger than the vector (4.1 kb) indicates that this fragment includes pIBI24 (2.9 kb). The approximately 2.0 kb fragment hybridizes with the chondroitinase probe, thereby serving to place one of the HindIII sites. Since there is a HindIII site in the polylinker, it too can be placed, leaving the last HindIII site to be placed by deduction.

Double digestion of the cloned approximately 10 kb NsiI fragment with EcoRV and EcoRI yields six fragments (of approximately 4.2 kb, 3.5 kb, 2.3 kb, 2.1 kb, 1 kb, and 0.4 kb), indicating the presence of two EcoRI sites -- one in the polylinker and one in the cloned P. vulgaris DNA. Southern hybridization reveals that the approximately 4.2 kb band in this double digest contains the chondroitinase I N-terminal coding sequence. Adding this information to the above data yields a preliminary restriction map for the subcloned approximately 10 kb NsiI fragment in pIBI24 (Figure 1).

It should be noted that, in further support of the placement and orientation of the chondroitinase I gene, in vitro chondroitinase I assays in which the activity of the enzyme based on measuring the release of unsaturated disaccharide from chondroitin sulfate C at 232 nm are carried out on a small number of samples. In one case, an aliquot of an overnight culture used to prepare LP^2 751 (ER1562 carrying cosmid DNA selected from the colony hybridizations) is found to exprss 0.12 units/ml of chondroitinase. In addition, one of the EcoRV-deletion constructions (to be described below) is grown overnight in the presence of ampicillin. This culture is then inoculated into fresh selective media either with or without isopropyl-beta-D-thiogalactopyranoside (IPTG) which is

expected to increase the level of transcription from the lac promoter present in pIBI24. The assay results of 0.29 units/ml of chondroitinase without and 0.36 units/ml with IPTG induction indicate that, even after the EcoRV deletion, the gene is still intact and possibly oriented in the same direction as that of the lac promoter.

Although the sizes of the fragments in the above discussion are approximate (especially the approximately 1 kb region between the EcoRI/NsiI in the polylinker and the nearest EcoRV site; in addition, there also might be another small EcoRV fragment that is still unmapped), overall they suggest that the approximately 4.2 kb EcoRV-EcoRI fragment contains the entire chondroitinase I gene. In order to facilitate the restriction mapping, an EcoRV deletion is constructed using the approximately 10 kb NsiI fragment cloned into pIBI24 ($LP^2$776). This DNA is digested with EcoRV, treated with calf intestinal alkaline phosphatase, and fractionated on an agarose gel. The largest (approximately 10kb) fragment is extracted from the gel and ligated together in the presence of a phosphorylated EcoRI linker. The resulting construction (LP^2 786) is next digested with EcoRI to yield three fragments. Although it is not completely separated from the pIBI24-containing, somewhat smaller fragment, an approximately 95% homogenous, approximately 4.2 kb EcoRI fragment is obtained after extraction from the gel. This EcoRI fragment is then used for DNA sequence analysis.

Example 5

DNA Sequence Analysis Of The Approximately 4.2 kb EcoRI Fragment

The approximately 10 kb NsiI fragment, cloned into pIBI24, is digested with EcoRV (as described above) and ligated together in the presence of EcoRI linkers. The net result of this construction is the deletion of approximately 5 kb of P. vulgaris DNA from this subcloned piece of DNA and the simultaneous introduction of another EcoRI site into the molecule. One hundred micrograms of this "EcoRV deletion" construction (LP^2 786) is digested with EcoRI and fractionated on an agarose gel. The desired approximately 4.2 kb fragment is eluted from the gel, precipitated and resuspended in 150 μl TE described above. One-third of this material is then ligated to itself (polymerized) and, after destruction of the DNA ligase by heating, the DNA is sonicated to generate random, small pieces suited to DNA sequence analysis.

The ends are rendered flush in a "fill-in" reaction mediated by the "Klenow fragment" (10; New England Biolabs, Beverly, MA) of the DNA polymerase I of E. coli, and then ligated into SmaI-cut and phosphatased M13mp19. This recombinant DNA is used to transform the male E. coli strain MV1190 and 500 of the phage plaques obtained are picked into SM buffer (NaCl, 100 mM, $MgSO_4$, 8 mM, Tris-HCl, pH 7.4, 50 mM and 0.01% gelatin) to serve as stocks for the infection of small (less than or equal to 10 ml) cultures that are then used for the isolation of single stranded template DNA.

DNA sequencing is carried out at elevated temperatures using Taq DNA polymerase and fluorescently-labeled oligonucleotide primers. The

data are collected using a Model 370A DNA sequencing system (Applied Biosystems, Foster City, CA). Sequence editing, overlap determinations and derivation of a consensus sequence are performed using a collection of computer programs obtained from the Genetics Computer Group at the University of Wisconsin (14). The resulting DNA sequence of this EcoRI fragment is 3980 nucleotides in length (SEQ ID NO:1). It is to be noted that the EcoRI site near the N-terminal coding sequence is derived from the linker ligated into this site; it is not present in the P. vulgaris chromosome. This position actually is an EcoRV site in the cloned cosmid DNA.

Translation of the DNA sequence into the putative amino acid sequence reveals a continuous open reading frame encoding of 1021 amino acids (SEQ ID NO:2), with a 24 residue signal sequence (SEQ ID NO:2, amino acids 1-24), followed by a 997 residue coding sequence for the mature (processed) chondroitinase I protein (SEQ ID NO:2, amino acids 25-1021). Computer analysis using the programs described above of this sequence predicts a molecular weight of 115,090.94 for the unprocessed protein, a molecular weight of 112,507.82 for the mature "110 kD" (transported) protein, 17,503.43 for the first 157 amino acids (the "18 kD" fragment) (SEQ ID NO:2, amino acids 25-181) and 95,022.40 for the remaining 840 amino acids (the "90 kD" fragment) (SEQ ID NO:2, amino acids 182-1021) and a molecular weight of 2601.14 for the 24-residue signal sequence. One notable feature of the amino acid composition is the absence of cysteine which could be important if the protein has to be re-folded at any point.

In the nucleotide sequence, it was noted above that there is a unique SphI restriction site

located approximately 230 bp beyond the end of the gene (SEQ ID NO:1, nucleotides 3414-3419), which presents a unique target site that can be manipulated to allow the facile movement of the gene to achieve the overall goal of expressing chondroitinase at high levels in E. coli. Although there are two recognition sites for ClaI (ATCGAT), one of them (SEQ ID NO:1, nucleotides 2702-2707) is embedded within the E. coli dam recognition sequence (GATC) (SEQ ID NO:1, nucleotides 2701-2704). The resulting adenine methylation by the dam-encoded enzyme blocks cleavage of this site by ClaI; therefore, there is, in effect, a "unique" ClaI site (SEQ ID NO:1, nucleotides 497-502) which is used, as described below, to reconstruct the chondroitinase I gene after the appropriate site-specific mutageneses are carried out.

Example 6

Site-specific Mutagenesis Of The Cloned P. vulgaris Chondroitinase I Gene

The site-specific mutagenesis method employed is based on that of Kunkel (15), using materials purchased from Bio-Rad, Melville, N.Y. (Muta-Gene™ In Vitro Mutagenesis Kit). In this procedure, the target DNA to be mutagenized is first cloned into an appropriate M13-derived vector. In this case, the recombinant molecule used (M13mp19 into which is cloned the approximately 1200 bp EcoRV-HindIII fragment as described above) encompasses the N-terminal coding region of the chondroitinase I gene. This recombinant phage is replicated in the E. coli host strain CJ236 (Bio-Rad), a male strain that carries the dut and ung alleles. The combination of these two mutations, dut (dUTPase) and ung (uracil-N-

glycosylase), results in the incorporation of some uracil, rather than thymine, residues into the DNA synthesized in this organism. Single stranded template is then isolated after propagation on CJ236 and an appropriate, mutagenic, synthetic oligonucleotide is annealed to this DNA.

This oligonucleotide serves as a primer for T7 DNA polymerase which copies the entire recombinant molecule. T4 DNA ligase is then used to seal the nick between the first residue of the mutagenic oligonucleotide and the last residue added *in vitro*. The newly synthesized DNA (containing the desired base changes) therefore does not contain uracil, while the template DNA does. Transformation of a non-mutant (with respect to the *dut* and *ung* alleles) male *E. coli* strain yields phage progeny that are primarily derived from the mutagenized strand synthesized *in vitro* as a result of the inactivation of the uracil-containing template strand.

In this specific case, four resuspended plaques (aliquots of which had been used for DNA sequencing which established the N-terminal coding region of the chondroitinase I gene and included another 110 bp "upstream" of the presumed translation initiation site (see above)) are used to infect the male host strain CJ236 (*dut ung*). Individual plaques are picked to 0.5 ml of phage dilution buffer (PDB). One picked plaque from each transformation is adsorbed to log phase CJ236 and the infected culture grown for 6.5 hours. The cells are pelleted by centrifugation, and the supernatant heated to 55°C for 30 minutes and then stored at 4°C. Single stranded DNA is isolated from 100 ml of each supernatant and resuspended in a total volume of 0.1 ml of TE.

The goal of the site-specific mutagenesis is

to modify the "ends" of this gene to allow it to be moved, precisely, into an appropriate high-level E. coli expression system. The target vector chosen (pET9-A; see above) is one derived from genetic regulatory elements present in the bacteriophage T7. In this sytem, there is a unique NdeI site (CATATG) that includes the translation initiation codon as well as a downstream BamHI site that, together, allow the direct, unidirectional, insertion of a gene encoding the protein that is to be expressed. These two sites are preceeded by a T7-specifc promoter sequence and trailed by a transcription terminator that functions with the T7 RNA polymerase. Accordingly, these two restriction sites (NdeI and BamHI) are introduced into the cloned gene for P. vulgaris chondroitinase I.

In order to introduce the NdeI site (containing the ATG initiation codon) both before the signal sequence as well as, in a second construction, before the coding sequence for the mature protein (thereby deleting the signal sequence), two synthetic oligonucleotides are designed and synthesized (purchased from BioSynthesis, Inc., Denton, TX). The first, designated oligonucleotide # 25 (SEQ ID NO:37), retains the signal sequence while the second, oligonucleotide #26 (SEQ ID NO:38), deletes the signal sequence and allows the direct expression of the mature chondroitinase I protein (which can have an additional methionine residue at the N-terminus (SEQ ID NO:5, amino acid number 1)).

The native sequence, including the predicted initiation codon, is presented on line 1 below while the mutagenic oligonucleotide #25 (which differs in the three nucleotides immediately upstream of the initiation codon) is presented on line 2:

1) 5'-GCCAGCGTTTCTAAGGAGAAAAATAATGCCGATATT-TCGTTTTACTGC-3' (SEQ ID NO:1, nucleotides 94-141)

2) 5'-GCCAGCGTTTCTAAGGAGAAAACATATGCCGATATT-TCGTTTTACTGC-3' (SEQ ID NO:37)

For the construction in which the signal sequence is deleted, the site-specific mutagenesis is carried out at the junction of the signal sequence and the start of the mature protein (line 3) using the mutagenic oligonucleotide # 26 (line 4) (which differs by six nucleotides, including the location of the initiation codon):

3) 5'-GCGCCTTATAACGCGATGGCAGCCACCAGCAATCCTG-3' (SEQ ID NO:1, nucleotides 170-206)

4) 5'-GCGCCTTATAACGCGCATATGGCCACCAGCAATCCTG-3' (SEQ ID NO:38)

The underlined GCC in line 3 corresponds to the codon for alanine which is the N-terminal amino acid for the mature, processed form of the P. vulgaris chondroitinase I.

In order for these oligonucleotides to be used, their 5'-ends need to be phosphorylated. Therefore, oligonucleotide # 25 (5 O.D. units) is resuspended with 0.5 ml of TE, while oligonucleotide # 26 (also 5 O.D. units) is resuspended in 0.65 ml TE to yield stocks that are approximately 20 nM, i.e., 20 pmole/μl. Three nanomoles (150 μl of stock solution) of each oligonucleotide are kinased in separate (0.35 ml) reactions containing 35 μl 10x ligase salts (New England Biolabs, Beverly, MA): 0.5 M Tris-HCl (pH

7.8), 0.1 M $MgCl_2$, 0.2 M dithiothreitol, 10 mM ATP, 0.5 mg/ml bovine serum albumin), 35 μl 0.1 M dithiothreitol, 10 μl (100 units) T4 polynucleotide kinase (New England Biolabs) and made up to volume with 120 μl TE. The reactions are incubated at 37°C for 40 minutes and the enzyme inactivated at 70°C for 20 minutes.

Template DNA (5 μl of the preparation described above) and phosphorylated mutagenic primer (approximately 2 pmole) are annealed in a 20 μl volume containing 20 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, and 50 mM NaCl. The sample is heated at 70°C for 45 minutes in a Perkin-Elmer/Cetus Thermalcycler™. The sample is then gradually cooled from 70°C to 25°C over a 45 minute period. The annealed mixture is placed on ice and the following components added: 2 μl of 10 X synthesis buffer (Bio-Rad): 5mM each of dATP, dGTP, dCTP, dTTP; 10 mM ATP; 100mM Tris-HCl (pH 7.4); 50 mM $MgCl_2$; 20 mM dithiothreitol), 2 μl of T4 DNA ligase (6 units) and 1 μl of T7 DNA polymerase (1 unit). These reactions are incubated for 5 minutes each at 0°C (on ice), 11°C, 25°C, and finally for 30 minutes at 37°C. The reactions are stopped by the addition of 75 μl of 10 mM Tris-HCl-10 mM EDTA (pH 8.0) and placed at -20°C.

After the mutagenized DNA is thawed, it is used to transform the male E. coli strain MV1190 (dut$^+$ ung$^+$). Individual plaques obtained are picked and single-stranded DNA is isolated and sequenced. For those cases in which the desired sequence changes are introduced, another aliquot of the resuspended plaque is used to infect strain MV1190, but in this case the intracellular, double-stranded replicative form of the recombinant DNA is isolated from the infected cell pellets using the Mini-Prep procedure referenced above.

Example 7

Reconstruction Of The Site-Specifically Mutagenized Chondroitinase I Gene And Its High-Level Expression In E. coli

Example 6 described the site-specific mutageneses that created an NdeI site immediately preceeding the signal sequence, as well as a second construction which placed the NdeI site adjacent to the triplet which codes for the N-terminal alanine found on the mature, processed P. vulgaris chondroitinase I gene. In each case, the ATG sequence of the NdeI recognition site (CATATG) can function as the translation initiation codon for the protein (either with or without the signal sequence).

In order to transfer these alterations from the M13 vector in which they were constructed, to the full chondroitinase I gene, the isolated replicative form is digested with KpnI and ClaI. The KpnI site is part of the M13mp19 polylinker, while the ClaI site is found approximately 490 bp from the end of the cloned fragment of the chondroitinase I gene. The restriction digestion products obtained are fractionated on a 4% NuSieve™ GTG agarose gel run in 1/2 X Tris-Acetate buffer (TAE). The appropriate approximately 500 bp band is extracted from the gel using Qiaex™. Similarly, plasmid DNA (LP^2 786) carrying the chondroitinase I gene is also digested with KpnI and ClaI and then fractionated on a 0.8% agarose gel run in 1/2 X TAE. In this case, the KpnI site is part of the polylinker of pIBI24, while the ClaI site corresponds to the one described above. (As stated above, there is a second ClaI site in the chondroitinase I gene, but it is not cleaved by ClaI because this site is apparently blocked by dam

methylation. The site-specific mutagenesis and reconstruction of the chondroitinase I gene were carried out before the entire nucleotide sequence was ascertained).

The approximately 7 kb fragment containing the pIBI24 vector and the large fragment of the chondroitinase I gene are isolated from the agarose gel by electroelution (11), followed by ethanol precipitation. This 7 kb fragment is then treated with calf intestinal alkaline phosphatase, extracted first with phenol-chloroform, then with chloroform, and then precipitated twice with ethanol and finally resuspended with 0.1 ml TE. The two isolated N-terminal encoding fragments (the two approximately 500 bp KpnI-ClaI pieces containing the two site-specifically mutagenized sequences, one with and one without the signal sequence) are each ligated to the approximately 7 kb fragment encompassing the remainder of the chondroitinase I gene and the pIBI24 vector. The ligase reaction is then used to transform the E. coli strain 294 and ampicillin resistant derivatives obtained. DNA is isolated from small (10 ml) cultures and digested with NdeI to verify the presence of this restriction site within the reconstructed DNA.

In order to remove the (apparent) P. vulgaris promoter and ribosome binding site, the modified chondroitinase I genes are isolated as approximately 4.5 kb NdeI-NsiI fragments and subcloned into a pBR322 variant in which the EcoRI site is first filled-in, then dephosphorylated, and finally a phosphorylated NsiI linker (New England Biolabs) inserted. The sequence of the linker used (TGCATGCATGCA) to place the NsiI site (ATGCAT) into pBR322 also includes an SphI site (GCATGC). In order to trim extra, non-coding DNA from the subcloned NdeI-

NsiI fragments, as well as to introduce a unique restriction site to be used later, plasmids (representing two clones each with the signal sequenc retained [LP^2 861 and LP^2 863] and two with the signal sequence deleted [LP^2 865 and LP^2 867]) containing the approximately 4500 bp NdeI-NsiI segments including the chondroitinase I gene are first digested with SphI, the ends "filled-in" with the "Klenow" fragment (11) of the E. coli DNA polymerase I and the resulting DNA fragments fractionated on an agarose gel (0.8% in 1/2 X TAE). The appropriate bands (approximately 5200 bp) are eluted from the gel using Qiaex™ and then treated with calf alkaline phosphatase. After the removal of this enzyme by phenol-chloroform and chloroform extractions, the DNA is precipitated twice and finally resuspended with 0.1 ml TE.

This DNA is then ligated in the presence of a phosphorylated BamHI linker and the mixture used to transform the E. coli strain 294. Six representative, ampicillin resistant colonies from each of the four constructions are grown in small (10 ml) cultures and plasmid DNA is isolated. Digestion of the DNA from the 24 clones examined with the enzymes NdeI and BamHI indicates which contain the BamHI site and, simultaneously, releases the approximately 3400 bp NdeI-BamHI fragment which contains the chondroitinase I gene. Seventeen clones (eight with and nine without the signal sequence) yield the desired fragment which is extracted from the agarose gel with Qiaex™.

These approximately 3.4 kb NdeI-Bam-HI chondroitinase I gene-containing fragments (both with and without the signal sequence) are then used to construct a high-level expression system. The expression vector used, pET-9A (9; Novagen), is derived from elements of the E. coli bacteriophage T7.

It contains an origin of replication derived from the Col E1 plasmid, a kanamycin resistance determinant, and the transcription and translation initiation determinants of the T7 gene 10. The naturally-occurring translation initiation codon for this gene is part of an NdeI site. This region is followed by a unique BamHI site and a T7 transcription terminator. A sample of this expression vector is digested with the restriction enzymes NdeI and BamHI, dephosphorylated with calf intestinal alkaline phosphatase, and purified by agarose gel electrophoresis. Each of the chondroitinase I gene fragments (both with and without the signal sequence) is ligated to the expression vector fragment. The resulting recombinant DNA mixture is used to transform the E. coli K-12 host, HMS174 (Novagen). Kanamycin-resistant colonies obtained are grown in small scale (10 ml) and plasmid DNA is isolated and examined to confirm the predicted structure.

Samples of these constructions are then used to transform the expression host BL21(DE3)/pLysS (10). This E. coli B strain carries the T7 RNA polymerase gene under lac control (and is therefore inducible by either lactose or IPTG) on a lambda phage integrated within the E. coli chromosome, as well as the Col E1-compatible plasmid pLysS. This latter replicon specifies resistance to chloramphenicol and contains the T7 lysozyme gene inserted into the tetracycline-resistance determinant of pACYC184 (ATCC 37033, American Type Culture Collection, Rockville, MD) in the "silent" orientation (read in the opposite direction relative to the tetracycline resistance gene). The T7 lysozyme is expressed at a relatively low level in this construction and serves as an inhibitor of the T7 RNA Polymerase (16), thereby

minimizing the basal-level expression of the gene to be overexpressed.

Derivatives of BL21(DE3)/pLysS carrying the chondroitinase I gene (with the signal sequence retained and which have been subjected to the site-directed mutagenesis described in Example 6 (SEQ ID NO:3)) in pET9-A are designated LL2084, LL2085, LL2086 and LL2087. They are not tested for expression of the chondroitinase I enzyme. The native chondroitinase I gene (with the signal sequence retained) (SEQ ID NO:1), which has not been subjected to site-directed mutagenesis, is inserted into a different expression host. Expression of the chondroitinase I enzyme is achieved.

One of the derivatives of BL21(DE3)/pLysS carrying the signal-less chondroitinase I gene which has been subjected to the site-directed mutagenesis described in Example 6 (SEQ ID NO:4) inserted into pET9-A, is designated LL2088, tested and used to establish a master cell bank. The insertion of the gene into pET9-A yields the plasmid designated pTM49-6. Samples of the E. coli B strain BL21(DE3)/pLysS carrying the plasmid pTM49-6 constitute the deposited strain ATCC 69234.

An overnight culture of this deposited strain is grown at 30°C in the presence of 40 μg/ml of kanamycin and 25 μg/ml of chloramphenicol. A 0.5 ml aliquot of this culture is used to inoculate 100 ml of a rich "expression" medium containing M9 salts (17) supplemented with 20 g/l tryptone, 10 g/l yeast extract, and 10 g/l dextrose in addition to the same level of kanamycin and chloramphenicol.

The culture is grown at 30°C to an appropriate density (a value of 1 at A_{600}) and then chondroitinase I expression is induced by the addition

of IPTG to a final concentration of 1 mM. After three hours, samples are taken, centrifuged, and the cell pellets frozen on dry ice prior to assay. The frozen pellets are thawed, resuspended in buffer and sonicated. A value of 56 units/ml is obtained (relative to the original culture volume), which indicates that this expression system is functional. A subsequent 10 liter fermentation under controlled conditions at a higher cell density yields a maximum value of approximately 600 units/ml of chondroitinase I. This represents a substantial improvement over fermentation of the original native P. vulgaris, which had not expressed chondroitinase I at a level above 2 units/ml.

Example 8

Method For The Isolation And Purification Of The Native Chondroitinase I Enzyme As Adapted To The Recombinant Enzyme

The native enzyme is produced by fermentation of a culture of P. vulgaris. The bacterial cells are first recovered from the medium and resuspended in buffer. The cell suspension is then homogenized to lyse the bacterial cells. Then a charged particulate such as 50 ppm Bioacryl (Toso Haas, Philadelphia, PA), is added to remove DNA, aggregates and debris from the homogenization step. Next, the solution is brought to 40% saturation of ammonium sulfate to precipitate out undesired proteins. The chondroitinase I remains in solution.

The solution is then filtered using a 0.22 micron SP240 filter (Amicon, Beverly, MA), and the retentate is washed using nine volumes of 40% ammonium sulfate solution to recover most of the enzyme. The

filtrate is concentrated and subjected to diafiltration with a sodium phosphate buffer using a 30 kD filter to remove salts and small molecules.

The filtrate containing chondroitinase I is subjected to cation exchange chromatography using a Cellufine™ cellulose sulfate column (Chisso Corporation, distributed by Amicon). At pH 7.2, 20 mM sodium phosphate, more than 98% of the chondroitinase I binds to the column. The native chondroitinase I is then eluted from the column using a 0 to 250 mM sodium chloride gradient, in 20 mM sodium phosphate buffer.

The eluted enzyme is then subjected to additional chromatography steps, such as anion exchange and hydrophobic interaction column chromatography. As a result of all of these procedures, chondroitinase I is obtained at a purity of 90-97% as measured by SDS-PAGE scanning (see above). However, the yield of the native protein is only 25-35%, determined as described above. This method also results in the cleavage of the approximately 110 kD chondroitinase I protein into a 90 kD and an 18 kD fragment. Nonetheless, the two fragments remain non-ionically bound and exhibit chondroitinase I activity.

When this procedure is repeated with lysed host cells carrying a recombinant plasmid encoding chondroitinase I, significantly poorer results are obtained. Less than 10% of the chondroitinase I binds to the cation exchange column at standard stringent conditions of pH 7.2, 20 mM sodium phosphate.

Under less stringent binding conditions of pH 6.8 and 5 mM phosphate, an improvement of binding with one batch of material to 60-90% is observed. However, elution of the recombinant protein with the NaCl gradient gives a broad activity peak, rather than

a sharp peak (see Figure 2). This indicates the product is heterogeneous. Furthermore, in subsequent fermentation batches, the recombinant enzyme binds poorly (1-40%), even using the less stringent binding conditions. Batches that bind poorly are not completely processed, so their overall recovery is not quantified.

Example 9

First Method For The Isolation And Purification Of Recombinant Chondroitinase I According To This Invention

As a first step, the host cells which express the recombinant chondroitinase I enzyme are homogenized to lyse the cells. This releases the enzyme into the supernatant.

In one embodiment of this invention, the supernatant is first subjected to diafiltration to remove salts and other small molecules. An example of a suitable filter is a spiral wound 30 kD filter made by Amicon (Beverly, MA). However, this step only removes the free, but not the bound form of the negatively charged molecules. The bound form of these charged species is removed by passing the supernatant through a strong, high capacity anion exchange resin-containing column. An example of such a resin is the Macro-Prep™ High Q resin (Bio-Rad, Melville, N.Y.). Other strong, high capacity anion exchange columns are also suitable. The negatively charged molecules bind to the column, while the enzyme passes through the column. It is also found that some unrelated, undesirable proteins also bind to the column.

Next, the eluate from the anion exchange column is directly loaded to a cation exchange resin-

containing column. Examples of such resins are the S-Sepharose™ (Pharmacia, Piscataway, N.J.) and the Macro-Prep™ High S (Bio-Rad). Each of these two resin-containing columns has SO_3^- ligands bound thereto in order to facilitate the exchange of cations. Other cation exchange columns are also suitable. The enzyme binds to the column and is then eluted with a solvent capable of releasing the enzyme from the column.

Any salt which increases the conductivity of the solution is suitable for elution. Examples of such salts include sodium salts, as well as potassium salts and ammonium salts. An aqueous sodium chloride solution of appropriate concentration is suitable. A gradient, such as 0 to 250 mM sodium chloride is acceptable, as is a step elution using 200 mM sodium chloride.

A sharp peak is seen in the sodium chloride gradient elution (Figure 3). The improvement in enzyme yield over the prior method is striking. The recombinant chondroitinase I enzyme is recovered at a purity of 99% at a yield of 80-90%.

The purity of the protein is measured by scanning the bands in SDS-PAGE gels. A 4-20% gradient of acrylamide is used in the development of the gels. The band(s) in each lane of the gel is scanned using the procedure described above.

These improvements are related directly to the increase in binding of the enzyme to the cation exchange column which results from first using the anion exchange column. In comparative experiments, when only the cation exchange column is used, only 1% of the enzyme binds to the column. However, when the anion exchange column is used first, over 95% of the enzyme binds to the column.

Example 10

Second Method For The Isolation And Purification Of Recombinant Chondroitinase I According To This Invention

In the second embodiment of this aspect of the invention, two additional steps are inserted in the method before the diafiltration step of the first embodiment. The supernatant is treated with an acidic solution, such as 1 M acetic acid, bringing the supernatant to a final pH of 4.5, to precipitate out the desired enzyme. The pellet is obtained by centrifugation at 5,000 x g for 20 minutes. The pellet is then dissolved in an alkali solution, such as 20-30 mM NaOH, bringing it to a final pH of 9.8. The solution is then subjected to the diafiltration and subsequent steps of the first embodiment of this invention.

In comparative experiments with the second embodiment of this invention, when only the cation exchange column is used, only 5% of the enzyme binds to the column. However, when the anion exchange column is used first, essentially 100% of the enzyme binds to the column. The second embodiment provides comparable enzyme purity and yield to the first embodiment of the invention.

Acid precipitation removes proteins that remain soluble; however, these proteins are removed anyway by the cation and anion exchange steps that follow (although smaller columns may be used). An advantage of the acid precipitation step is that the sample volume is decreased to about 20% of the original volume after dissolution, and hence can be handled more easily on a large scale. However, the additional acid precipitation and alkali dissolution

steps of the second embodiment mean that the second embodiment is more time consuming than the first embodiment. On a manufacturing scale, the marginal improvements in purity and yield provided by the second embodiment may be outweighed by the simpler procedure of the first embodiment, which still provides highly pure enzyme at high yields.

The high purity of the recombinant enzyme obtained by the two embodiments of this invention is depicted in Figure 4. A single sharp band is seen in the SDS-PAGE gel photograph: Lane 1 is the enzyme using the method of the first embodiment; Lane 2 is the enzyme using the method of the second embodiment; Lane 3 represents the supernatant from the host cell prior to purification -- many other proteins are present; and Lane 4 represents molecular weight standards.

Example 11

Site-Specific Mutagenesis Of A Fragment Encoding The N-Terminal Region Of Chondroitinase II

The approach taken in the case of the chondroitinase II gene is to modify the naturally-occurring ATG initiation codon to embed it within an NdeI site. This results in a construction in which the signal peptide is retained, such that the expressed gene is processed and secreted to yield the mature native enzyme structure that has a leucine residue at the N-terminus. The mutagenized bases are upstream of the coding region.

The method used for this site-specific alteration is that described above for the expression of the chondroitinase I gene and is based on the work of Kunkel (15) using the Muta-Gene™ In Vitro

Mutagenesis Kit Version 2 (Bio-Rad, Melville, N.Y.). In this procedure, the target DNA to be mutagenized is first cloned into a suitable M13-derived vector to generate single-stranded DNA. This recombinant phage is replicated in the E. coli host strain CJ236 (Bio-Rad), a male strain that carries the dut and ung alleles. The combination of these two mutations, dut (duTPase) and ung (uracil-N-glycosylase), results in the incorporation of some uracil, rather than thymine, residues into the DNA synthesized in this organism. Single-stranded template is then isolated after propagation on CJ236 and the appropriate mutagenic, synthetic oligonucleotide (SEQ ID NO:41) is annealed to this DNA.

This oligonucleotide serves as a primer for T7 DNA polymerase which copies the entire recombinant molecule. T4 DNA ligase is then used to seal the nick between the first residue of the mutagenic oligonucleotide and the last residue added in vitro. The newly synthesized DNA (containing the desired base changes) therefore does not contain uracil while the template DNA (with the native sequences) does. Transformation of a non-mutant (with respect to the ung and dut alleles) male E. coli strain yields phage progeny that are primarily derived from the mutagenized strand synthesized in vitro as a result of the inactivation of the uracil-containing template strand.

In this specific case, the fragment to be cloned for the mutagenesis is a MunI-EcoRI fragment that spans the region between nucleotides 2943 to 3980 (SEQ ID NOS:1 and 39). The DNA digested to obtain this fragment is designated LP^{2}783. This plasmid is constructed in the same way as LP^{2}786 (described in Example 4), except that a HindIII linker is inserted

into the EcoRV deletion of LP²776 rather than the EcoRI linker. This MunI-EcoRI fragment is ligated into the unique EcoRI site of LP²941, an M13mp19 derivative in which the normal polylinker is replaced with that found in the plasmid vector pNEB193 (New England Biolabs, Beverly MA). The four base overhang produced by MunI digestion can be ligated to an EcoRI site, but the resulting recombinant sequence cannot be digested by either enzyme. The EcoRI digested LP²941 is also dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis IN) prior to gel purification and use.

The ligated DNA mixture is used to infect the male E. coli strain MV1190 and the plaques obtained are picked to 0.5 ml. of SM buffer and the phage allowed to elute by diffusion. These are then used to infect 10 ml. cultures of MV1190 and grown overnight. The cultures are centrifuged and the pellets used for the isolation of the double-stranded replicative forms of the recombinant virus. The supernatants, which contain the corresponding phage particles, are stored under refrigeration until needed. The orientation of the cloned fragment is determined by digestion of the replicative form DNA and HindIII, because there is one site within the polylinker and a second, aymmetrically placed site (SEQ ID NOS:1 and 39, nucleotides 3326-3331) within the above MunI-EcoRI fragment.

Once the desired orientation is identified, the corresponding phage-containing supernatant is serially diluted, used to infect the E. coli strain CJ236, and then plated to obtain single plaques which are picked and eluted as above. One of these is then used to infect CJ236 and another 10 ml culture grown and the single-stranded DNA is isolated from the

phage-containing supernatant using Qiaex™ columns and materials and methods recommended by the manufacturer (Qiagen, Chatsworth, CA) and finally resuspended in a volume of 0.01 ml. The recombinant phage are grown on CJ236 (dut⁻ ung⁻) for two rounds in order to maximize the accumulation of uracil residues in the template and strand prior to the actual site-specific mutagenesis.

The mutagenic oligonucleotide used is obtained from Bio-Synthesis (Denton, TX) and has the following sequence:

5'-ATT-TGC-AGG-AAA-TCT-GCA-TAT-GCT-AAT-AAA-AAA-CCC-3' (SEQ ID NO:41)

This sequence differs from the corresponding region of SEQ ID NOS:1 and 39 in that an AT sequence (base pairs 3235 and 3236) is replaced by a CA sequence which creates the desired NdeI sequence (CATATG) at the start of the presumed leader sequence for the chondroitinase II gene. One optical density unit of this oligonucleotide is dissolved in 0.46 ml. of TE 7.4 (0.01M TrisHCl, pH 7.8-0.001M EDTA, pH 8.0), yielding an oligonucleotide concentration of approximately 6 pmol/μl. Three hundred picomoles of this oligonucleotide are phosphorylated in a 0.1 ml reaction containing 0.05 M TrisHCl, pH 7.8, 0.01 M $MgCl_2$, 0.02M dithiothreitol, 0.001 M ATP, 25 μg/ml bovine serum albumin and 100 units of T4 polynucleotide kinase (New England Biolabs) at 37°C for 30 minutes, followed by incubation at 75° for 20 minutes to inactivate the enzyme. The phosphorylated oligonucleotide is then stored frozen at -20° at a concentration of approximately 3 pmoles/μl.

For the site-specific mutagenesis, 1 μl (3

pmole) of the mutagenic oligonucleotide is mixed with 6 μl of the single-stranded DNA prepared above in a 10 μl volume of 0.02 M TrisHCl, pH 7.4, 0.002 M $MgCl_2$, 0.05 M NaCl. The oligonucleotide is annealed to this template by first incubating the sample at 70°C for 5 minutes and then cooling this sample at 25°C over a 45 minute period in a DNA Thermal Cycler™ (Perkin-Elmer Cetus/Norwalk, CT). The sample is maintained at 25°C for another 5 minutes before being cooled to 20°C and finally transferred to an ice bath.

The annealed primer is then extended after the addition of 1 μl of 10X synthesis buffer (Bio-Rad; containing 0.005 M of each of the dNTP's, 0.01 M ATP, 0.1 M TrisHCl, pH 7.4, 0.05 M $MgCl_2$, 0.02 M DTT). One μl of T4 DNA ligase (3 units/μl Bio-Rad) and 1 μl of T7 DNA polymerase (0.5 units/μl Bio-Rad). The *in vitro* DNA synthesis is carried out on ice for 5 minutes, at 11°C for ten minutes, and at 37°C for 30 minutes prior to transfer to ice.

This sample is used directly to transform the male *E. coli* host MV1190 (*dut*$^+$ *ung*$^+$) and the resulting plaques, containing the site-specifically mutagenized phage, are obtained, picked and eluted as described above. Aliquots of these phage stocks are used in infect 10 ml. cultures of MV1190 and allowed to grow overnight. The cultures are centrifuged and the replicative forms of the recombinant phage are isolated using Qiaex™ columns and methods recommended by the manufacturer (Qiagen, Chatsworth CA). The DNA isolated is resuspended in 0.1 ml of TE 7.4. Initial digestions of a portion of each of these DNA samples with *NdeI* reveals that at least four appeared to have acquired a new *NdeI* site, indicate that the site-specific mutagenesis is successful. Consequently, larger samples of these four clones (0.04 ml each) are

digested with NdeI and EcoRI and fractionated on a 1.4% agarose gel run in a Tris-acetate-EDTA buffer system.

The desired approximately 740 base pair fragment is observed in each case and this band is excised from each pattern. The four samples are then combined and the DNA extracted from the gel using a Qiaex™ resin and buffers according to the manufacturer's recommendations (Qiagen, Chatsworth CA) and resuspended in 0.05 ml. of TE, pH 7.4. This isolated, site-specifically mutagenized N-terminal coding region of the cloned P. vulgaris gene for the chondroitinase II gene is then subcloned into the plasmid pNEB193 (New England Biolabs, Beverly MA) between the (dephosphorylated) unique NdeI and EcoRI sites present in this plasmid. After transformation of the E. coli host strain 294, 10 ml cultures derived from the individual transformants are grown and the recombinant plasmid DNA isolated as above. The DNA sample from one of the positive clones is designated m#15-5712. This sample represents the modified N-terminal region that is to be joined to the C-terminal coding region for the chondroitinase II gene, which is described in Example 12.

Example 12

Isolation, Characterization And DNA Sequence Analysis Of A Fragment Encoding The C-terminal Region Of Chondroitinase II

The DNA sequence contained in SEQ ID NO: 39 indicates that chondroitinase II is encoded by a region that is downstream of that for chondroitinase I. This information is derived from a portion of a 10 kilobase NsiI fragment of P. vulgaris that is

subcloned originally from a cosmid clone designated LP²751. The combination of the DNA sequencing and the restriction map in Figure 1 reveals that the chondroitinase II coding region initiates to the "left" of the EcoRI site that lies within the P. vulgaris derived DNA and proceeds toward the NsiI site at the "right" end of the fragment depicted in Figure 1. Therefore, this restriction map should be expanded to the "right" to find a suitable fragment that will include the C-terminal coding region for the chondroitinase II gene.

Digestion of LP²751 reveals three EcoRI fragments of approximately 20 kb, 13 kb, and 10 kb, and indicates that there are three EcoRI sites within LP²751. Because there are two EcoRI sites that bracket the cloning site, the conclusion is that there is one EcoRI site within the cloned P. vulgaris DNA in this clone. Furthermore, since the approximately 13 kb fragment corresponds to the size of the cosmid vector per se, this unique EcoRI site lies between the approximately 20 kb and the approximately 10 kb fragments noted above. Since it is known that the entire coding region for chondroitinase I, as well as the N-terminal coding region for chondroitinase II, are both contained within the approximately 10 kb NsiI fragment, restriction digestions that compare the patterns obtained among the cloned 10 kb NsiI (present in the recombinant plasmid designated LP²770) and gel-purified samples of the above approximately 20 kb EcoRI and approximately 10 kb EcoRI fragments indicate which of these EcoRI fragments contain the chondroitinase I coding sequence and, therefore by deduction, which will carry the C-terminal coding region for chondroitinase II. Consequently, digestions are carried using the restriction enzymes

AflIII, ClaI, EcoRV, and HindIII each of which has been noted by Applicants to yield eight to ten fragments upon digestion of the original cosmid clone designated LP²751.

The recombinant molecule carrying the subcloned approximately 10 kb NsiI fragment (LP²770) and the individually gel-purified approximately 20 kb EcoRI and approximately 10 kb EcoRI fragments are digested with each of these enzymes to yield patterns of fragments that are compared. These digestions reveal that the approximately 20 kb EcoRI and the LP²770 patterns have a number of fragments in common. This indicates that the chondroitinase I gene and the N-terminal coding region of the chondroitinase II gene are contained within the larger EcoRI fragment and, therefore, the C-terminal coding region for the chondroitinase II gene is on the approximately 10 kb EcoRI fragment.

The approximately 10 kb EcoRI fragment is cloned into the unique EcoRI site of the derivative of pNEB193 (New England Biolabs, Beverly MA) that is designated lacpoΔ pNEB193. This vector carries two deletions relative to the parental molecule pNEB193. The first removes the sequences between the unique NdeI and EcoRI sites, retaining the EcoRI site but removing the NdeI site (and one of the two PvuII sites). The second deletion removes the region between the HindIII site at the other end of the polylinker and the (now unique) PvuII site, maintaining the HindIII site, while removing the PvuII site. The recombinant DNA molecule carrying the subcloned approximately 10 kb EcoRI fragment in the vector lacpoΔ pNEB193 is designated LP²1263. The orientation of the 112 kD C-terminal coding region within LP²1263 is determined by restriction enzyme

mapping. The results indicate that this region is positioned so as to proceed from the EcoRI site (defined as the "left" end) toward the HindIII site at the other end of the polylinker. Similarly, unique restriction sites for SmaI, XhoI, NocI and NdeI are found approximately 2.6, 4.6, 5.8 and 8.5 kb from the "left" end of the approximately 10 kb EcoRI fragment. Digestion of LP²1263 with SmaI, therefore, deletes a downstream region of approximately 7.4 kb from the site within the cloned P. vulgaris DNA to the second site within the polylinker region, leaving approximately 2.6 kb which should be enough to encode the missing region of the chondroitinase II gene. This construction also "places" a BamHI site (present in the polylinker) downstream of the coding region for the chondroitinase II gene. This recombinant DNA molecule which carries the chondroitinase II gene from the EcoRI site to (and presumably just beyond) the termination codon for this gene has been designated m#25-5712.

DNA sequence analysis is initiated on the approximately 10 kb EcoRI fragment derived from LP²1263 and is completed after the assembly of the intact gene for chondroitinase II. The materials and methods for the DNA sequencing of this fragment are essentially the same as those used for the approximately 4 kb fragment containing the gene for chondroitinase I. Random fragments are derived from this approximately 10 kb EcoRI fragment by self-ligating the DNA and then fragmenting the polymerized DNA by sonication as well as by partial digestion with the restriction enzymes Sau3A or MseI. These pieces are then eventually cloned into M13 derived vectors and the single-stranded recombinant molecules sequenced using the standard protocols described above.

Finally, with the two set of sequence data available, an approximately 300 base-pair BclI fragment is identified that is predicted to contain the EcoRI site that is the junction between the two P. vulgaris fragments of approximately 20 kb and approximately 10 kb obtained by digestion with EcoRI. This small fragment is sequenced in both directions to verify the nucleotide sequence through this junction point used in the constructions described below.

Example 13

Assembly Of The Entire Site-Specifically Mutagenized Gene For Chondroitinase II

During the DNA sequencing, the molecule designated m#25-5712 is digested with EcoRI and BamHI. This releases a DNA fragment of approximately 2.6 kb. Similarly, the construction designated m#15-5712 is digested with EcoRI and BamHI and then dephosphorylated prior to purification by gel electrophoresis. The latter molecule therefore carries the N-terminal coding region of the chondroitinase II gene from the ATG initiation codon (now present as part of an NdeI site from the site-specific mutagenesis) to the EcoRI site.

These two fragments are ligated and then the mixture used to transform the E. coli strain 294. Plasmid DNA is isolated from the transformants and positive clones identified. Restriction digestion with NdeI and BamHI releases the desired fragment encoding the chondroitinase II gene (SEQ ID NO:39, nucleotides 3235-6518, followed by 14 nucleotides derived from the polylinker, which includes a BamHI site). This fragment is then ligated to the expression vector pET9A (Novagen, Madison, WI)

described in the expression of the chondroitinase I gene.

The coding region of the chondroitinase II gene includes nucleotides 3238-6276 of the SEQ ID NO: 39, which encodes 1013 amino acids (SEQ ID NO:40). Of this region, nucleotides 3238-3306 encode the 23 amino acid signal peptide (SEQ ID NO:40, amino acids 1-23), while nucleotides 3307-6276 encode the mature 990 amino acid chondroitinase II protein (SEQ ID NO:40, amino acids 24-1013).

Restriction analysis with four enzymes of the region spanning both chondroitinase genes and flanking sequences thereof reveals the following restriction sites:

Enzyme	Nucleotide	Enzyme	Nucleotide
EcoRI	2	MunI	4510
HindIII	2046	HindIII	4530
MunI	2904	MunI	5176
MunI	2943	HindIII	5427
HindIII	3326	SmaI	6515
EcoRI	3974		

In addition, restriction analysis with Sau3AI reveals a multiplicity of sites, including those at SEQ ID NO:39, nucleotides 212, 602, 890, 1042, 1181, 1241, 1442, 1505, 1746, 2330, 2363, 2701, 2705, 2920, 3697, 3708, 3745, 3868, 4087, 4800, 4872, 5565, 5635, 5860, 6058 and 6467.

One of the recombinant molecules (the chondroitinase II gene inserted into pET9A) obtained in this experiment is grown in large scale (0.5 liter) and the expression system containing the chondroitinase II gene isolated and designated LP^{2}1359. An aliquot of this DNA is used to transform the expression host BL21(DE3)/pLysS described in the

expression of the chondroitinase I gene. The resulting strain is designated TD112 and is used for large-scale fermentation and isolation of the chondroitinase II enzyme.

A fermentation at a 10 liter scale carried out with this E. coli strain containing the plasmid expressing the chondroitinase II protein, provides a maximum chondroitinase II titer of approximately 0.3 mg/ml, which is approximately 25 times that of the approximately 0.012 mg/ml obtained from the native P. vulgaris fermentation process for chondroitinase II.

Example 14

First Method For The Isolation And Purification Of Recombinant Chondroitinase II According To This Invention

The initial part of this method is the same as that used for the recombinant chondroitinase I enzyme. As a first step, the host cells which express the recombinant chondroitinase II enzyme are homogenized to lyse the cells. This releases the enzyme into the supernatant.

In one embodiment of this invention, the supernatant is first subjected to diafiltration to remove salts and other small molecules. An example of a suitable filter is a spiral wound 30 kD filter made by Amicon (Beverly, MA). However, this step only removes the free, but not the bound form of the negatively charged molecules. The bound form of these charged species is removed by passing the supernatant (see the SDS-PAGE gel depicted in Figure 5, lane 1) through a strong, high capacity anion exchange resin-containing column. An example of such a resin is the Macro-Prep™ High Q resin (Bio-Rad, Melville, N.Y.).

Other strong, high capacity anion exchange columns are also suitable. The negatively charged molecules bind to the column, while the enzyme passes through the column with approximately 90% recovery of the enzyme. It is also found that some unrelated, undesirable proteins also bind to the column.

Next, the eluate from the anion exchange column (Figure 5, lane 2) is directly loaded to a cation exchange resin-containing column. Examples of such resins are the S-Sepharose™ (Pharmacia, Piscataway, N.J.) and the Macro-Prep™ High S (Bio-Rad). Each of these two resin-containing columns has SO_3^- ligands bound thereto in order to facilitate the exchange of cations. Other cation exchange columns are also suitable. The enzyme binds to the column, while a significant portion of contaminating proteins elute unbound.

At this point, the method diverges from that used for the chondroitinase I protein. Instead of eluting the protein with a non-specific salt solution capable of releasing the enzyme from the cation exchange column, a specific elution using a solution containing chondroitin sulfate is used. A 1% concentration of chondroitin sulfate is used; however, a gradient of this solvent is also acceptable. The specific chondroitin sulfate solution is preferred to the non-specific salt solution because the recombinant chondroitinase II protein is expressed at levels approximately several-fold lower than the recombinant chondroitinase I protein; therefore, a more powerful and selective solution is necessary in order to obtain a final chondroitinase II product of a purity equivalent to that obtained for the chondroitinase I protein.

The cation exchange column is next washed

with a phosphate buffer, pH 7.0, to elute unbound proteins, followed by washing with borate buffer, pH 8.5, to elute loosely bound contaminating proteins and to increase the pH of the resin to that required for the optimal elution of the chondroitinase II protein using the substrate, chondroitin sulfate.

Next, a 1% solution of chondroitin sulfate in water, adjusted to pH 9.0, is used to elute the chondroitinase II protein, as a sharp peak (recovery 65%) and at a high purity of approximately 95% (Figure 5, lane 3). However, the chondroitin sulfate has an affinity for the chondroitinase II protein which is stronger than its affinity for the resin of the column, and therefore the chondroitin sulfate co-elutes with the protein. This ensures that only protein which recognizes chondroitin sulfate is eluted, which is desirable, but also means that an additional process step is necessary to separate the chondroitin sulfate from the chondroitinase II protein.

In this separation step, the eluate is adjusted to pH 7.0 and is loaded as is onto an anion exchange resin-containing column, such as the Macro-Prep™ High Q resin. The column is washed with a 20 mM phosphate buffer, pH 6.8. The chondroitin sulfate binds to the column, while the chondroitinase II protein flows through in the unbound pool with greater than 95% recovery. At this point, the protein is pure, except for the presence of a single minor contaminant of approximately 37 kD (Figure 5, lanes 4 and 6). The contaminant may be a breakdown product of the chondroitinase II protein.

This contaminant is effectively removed by a crytallization step. The eluate from the anion exchange column is concentrated to 15 mg/ml protein

using an Amicon stirred cell with a 30 kD cutoff. The solution is maintained at 4°C for several days to crystallize out the pure chondroitinase II protein. The supernatant contains the 37 kD contaminant (Figure 5, lane 7). Centrifugation causes the crystals to form a pellet, while the supernatant with the 37 kD contaminant is removed by pipetting, and the crystals washed twice with water. After the first wash, some of the contaminant remains (Figure 5, lane 8), but after the second wash, only the chondroitinase II protein is visible (Figure 5, lane 9). The washed crystals are redissolved in water and exhibit a single protein band on SDS-PAGE, with a purity of greater than 99% (Figure 5, lane 10).

Example 15

Second Method For The Isolation And Purification Of Recombinant Chondroitinase II According To This Invention

In the second embodiment of this aspect of the invention, two additional steps are inserted in the method for purifying the chondroitinase II enzyme before the diafiltration step of the first embodiment. The supernatant is treated with an acidic solution, such as 1 M acetic acid, bringing the supernatant to a final pH of 4.5, to precipitate out the desired enzyme. The pellet is obtained by centrifugation at 5,000 x g for 20 minutes. The pellet is then dissolved in an alkali solution, such as 20-30 mM NaOH, bringing it to a final pH of 9.8. The solution is then subjected to the diafiltration and subsequent steps of the first embodiment of this aspect of the invention.

Bibliography

1. Yamagata, T., et al., J. Biol. Chem., 243, 1523-1535 (1968).

2. Kikuchi, H., et al., U.S. Patent Number 5,198,355.

3. Brown, M. D., U.S. Patent Number 4,696,816.

4. Hageman, G. S., U.S. Patent Number 5,292,509.

5. Malitschek, B., and Schartl, M., Biotechniques, 7, 177-178 (1991).

6. Sanger, F., et al., Proc. Natl. Acad. Sci. USA, 74, 5463-5467 (1977).

7. Innis, M. A., and Gelfand, D. H., "Optimization of PCRs", pages 3-12 in PCR Protocols, A Guide to Methods and Applications, Academic Press, New York, N.Y. (1990).

8. Southern, E., J. Mol. Biol., 98, 503-517 (1975).

9. Studier, F. W., et al., Methods in Enzymology, 185, 60-89 (1990).

10. Studier, F. W., and Moffatt, B. A., J. Mol. Biol., 189, 113-130 (1986); Moffatt, B. A., and Studier, F. W., Cell, 49, 221-227 (1987).

11. Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

12. Clark, Nucleic Acids Research, 16, 9677 (1988).

13. Yanisch-Perron, C., et al., Gene, 3, 103-119 (1985).

14. Devereaux, et al., Nucleic Acids Research, 12, 387-395 (1984).

15. Kunkel, T. A., Proc. Natl. Acad. Sci.,

USA, 82, 488-492 (1985).

16. Studier, F. W., J. Mol. Biol., 219, 37-44 (1991).

17. Miller, J. H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1972).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: American Cyanamid Company

(ii) TITLE OF INVENTION: Cloning And Expression Of The Chondroitinase I and II Genes From P. Vulgaris

(iii) NUMBER OF SEQUENCES: 41

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: American Cyanamid Company
(B) STREET: One Cyanamid Plaza
(C) CITY: Wayne
(D) STATE: New Jersey
(E) COUNTRY: U.S.A.
(F) ZIP: 07470-8426

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US94/
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Gordon, Alan M.
(B) REGISTRATION NUMBER: 30,637
(C) REFERENCE/DOCKET NUMBER: 31,726-00/PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 201-831-3244
(B) TELEFAX: 201-831-3305

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3980 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 119..3181

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GGAATTCCAT CACTCAATCA TTAAATTTAG GCACAACGAT GGGCTATCAG CGTTATGACA      60

AATTTAATGA AGGACGCATT GGTTTCACTG TTAGCCAGCG TTTCTAAGGA GAAAAATA      118

ATG CCG ATA TTT CGT TTT ACT GCA CTT GCA ATG ACA TTG GGG CTA TTA      166
Met Pro Ile Phe Arg Phe Thr Ala Leu Ala Met Thr Leu Gly Leu Leu
  1               5                  10                  15

TCA GCG CCT TAT AAC GCG ATG GCA GCC ACC AGC AAT CCT GCA TTT GAT      214
Ser Ala Pro Tyr Asn Ala Met Ala Ala Thr Ser Asn Pro Ala Phe Asp
            20                  25                  30

CCT AAA AAT CTG ATG CAG TCA GAA ATT TAC CAT TTT GCA CAA AAT AAC      262
Pro Lys Asn Leu Met Gln Ser Glu Ile Tyr His Phe Ala Gln Asn Asn
        35                  40                  45

CCA TTA GCA GAC TTC TCA TCA GAT AAA AAC TCA ATA CTA ACG TTA TCT      310
Pro Leu Ala Asp Phe Ser Ser Asp Lys Asn Ser Ile Leu Thr Leu Ser
    50                  55                  60

GAT AAA CGT AGC ATT ATG GGA AAC CAA TCT CTT TTA TGG AAA TGG AAA      358
Asp Lys Arg Ser Ile Met Gly Asn Gln Ser Leu Leu Trp Lys Trp Lys
65                  70                  75                  80

GGT GGT AGT AGC TTT ACT TTA CAT AAA AAA CTG ATT GTC CCC ACC GAT      406
Gly Gly Ser Ser Phe Thr Leu His Lys Lys Leu Ile Val Pro Thr Asp
                85                  90                  95

AAA GAA GCA TCT AAA GCA TGG GGA CGC TCA TCT ACC CCC GTT TTC TCA      454
Lys Glu Ala Ser Lys Ala Trp Gly Arg Ser Ser Thr Pro Val Phe Ser
            100                 105                 110

TTT TGG CTT TAC AAT GAA AAA CCG ATT GAT GGT TAT CTT ACT ATC GAT      502
Phe Trp Leu Tyr Asn Glu Lys Pro Ile Asp Gly Tyr Leu Thr Ile Asp
        115                 120                 125

TTC GGA GAA AAA CTC ATT TCA ACC AGT GAG GCT CAG GCA GGC TTT AAA      550
Phe Gly Glu Lys Leu Ile Ser Thr Ser Glu Ala Gln Ala Gly Phe Lys
    130                 135                 140

GTA AAA TTA GAT TTC ACT GGC TGG CGT GCT GTG GGA GTC TCT TTA AAT      598
Val Lys Leu Asp Phe Thr Gly Trp Arg Ala Val Gly Val Ser Leu Asn
145                 150                 155                 160

AAC GAT CTT GAA AAT CGA GAG ATG ACC TTA AAT GCA ACC AAT ACC TCC      646
Asn Asp Leu Glu Asn Arg Glu Met Thr Leu Asn Ala Thr Asn Thr Ser
                165                 170                 175

TCT GAT GGT ACT CAA GAC AGC ATT GGG CGT TCT TTA GGT GCT AAA GTC      694
Ser Asp Gly Thr Gln Asp Ser Ile Gly Arg Ser Leu Gly Ala Lys Val
            180                 185                 190

GAT AGT ATT CGT TTT AAA GCG CCT TCT AAT GTG AGT CAG GGT GAA ATC      742
Asp Ser Ile Arg Phe Lys Ala Pro Ser Asn Val Ser Gln Gly Glu Ile
        195                 200                 205

TAT ATC GAC CGT ATT ATG TTT TCT GTC GAT GAT GCT CGC TAC CAA TGG      790
Tyr Ile Asp Arg Ile Met Phe Ser Val Asp Asp Ala Arg Tyr Gln Trp
    210                 215                 220

TCT GAT TAT CAA GTA AAA ACT CGC TTA TCA GAA CCT GAA ATT CAA TTT      838
Ser Asp Tyr Gln Val Lys Thr Arg Leu Ser Glu Pro Glu Ile Gln Phe
```

```
225                 230                 235                 240

CAC AAC GTA AAG CCA CAA CTA CCT GTA ACA CCT GAA AAT TTA GCG GCC      886
His Asn Val Lys Pro Gln Leu Pro Val Thr Pro Glu Asn Leu Ala Ala
                245                 250                 255

ATT GAT CTT ATT CGC CAA CGT CTA ATT AAT GAA TTT GTC GGA GGT GAA      934
Ile Asp Leu Ile Arg Gln Arg Leu Ile Asn Glu Phe Val Gly Gly Glu
            260                 265                 270

AAA GAG ACA AAC CTC GCA TTA GAA GAG AAT ATC AGC AAA TTA AAA AGT      982
Lys Glu Thr Asn Leu Ala Leu Glu Glu Asn Ile Ser Lys Leu Lys Ser
        275                 280                 285

GAT TTC GAT GCT CTT AAT ATT CAC ACT TTA GCA AAT GGT GGA ACG CAA     1030
Asp Phe Asp Ala Leu Asn Ile His Thr Leu Ala Asn Gly Gly Thr Gln
    290                 295                 300

GGC AGA CAT CTG ATC ACT GAT AAA CAA ATC ATT ATT TAT CAA CCA GAG     1078
Gly Arg His Leu Ile Thr Asp Lys Gln Ile Ile Ile Tyr Gln Pro Glu
305                 310                 315                 320

AAT CTT AAC TCC CAA GAT AAA CAA CTA TTT GAT AAT TAT GTT ATT TTA     1126
Asn Leu Asn Ser Gln Asp Lys Gln Leu Phe Asp Asn Tyr Val Ile Leu
                325                 330                 335

GGT AAT TAC ACG ACA TTA ATG TTT AAT ATT AGC CGT GCT TAT GTG CTG     1174
Gly Asn Tyr Thr Thr Leu Met Phe Asn Ile Ser Arg Ala Tyr Val Leu
            340                 345                 350

GAA AAA GAT CCC ACA CAA AAG GCG CAA CTA AAG CAG ATG TAC TTA TTA     1222
Glu Lys Asp Pro Thr Gln Lys Ala Gln Leu Lys Gln Met Tyr Leu Leu
        355                 360                 365

ATG ACA AAG CAT TTA TTA GAT CAA GGC TTT GTT AAA GGG AGT GCT TTA     1270
Met Thr Lys His Leu Leu Asp Gln Gly Phe Val Lys Gly Ser Ala Leu
    370                 375                 380

GTG ACA ACC CAT CAC TGG GGA TAC AGT TCT CGT TGG TGG TAT ATT TCC     1318
Val Thr Thr His His Trp Gly Tyr Ser Ser Arg Trp Trp Tyr Ile Ser
385                 390                 395                 400

ACG TTA TTA ATG TCT GAT GCA CTA AAA GAA GCG AAC CTA CAA ACT CAA     1366
Thr Leu Leu Met Ser Asp Ala Leu Lys Glu Ala Asn Leu Gln Thr Gln
                405                 410                 415

GTT TAT GAT TCA TTA CTG TGG TAT TCA CGT GAG TTT AAA AGT AGT TTT     1414
Val Tyr Asp Ser Leu Leu Trp Tyr Ser Arg Glu Phe Lys Ser Ser Phe
            420                 425                 430

GAT ATG AAA GTA AGT GCT GAT AGC TCT GAT CTA GAT TAT TTC AAT ACC     1462
Asp Met Lys Val Ser Ala Asp Ser Ser Asp Leu Asp Tyr Phe Asn Thr
        435                 440                 445

TTA TCT CGC CAA CAT TTA GCC TTA TTA TTA CTA GAG CCT GAT GAT CAA     1510
Leu Ser Arg Gln His Leu Ala Leu Leu Leu Leu Glu Pro Asp Asp Gln
    450                 455                 460

AAG CGT ATC AAC TTA GTT AAT ACT TTC AGC CAT TAT ATC ACT GGC GCA     1558
Lys Arg Ile Asn Leu Val Asn Thr Phe Ser His Tyr Ile Thr Gly Ala
465                 470                 475                 480
```

```
TTA ACG CAA GTG CCA CCG GGT GGT AAA GAT GGT TTA CGC CCT GAT GGT      1606
Leu Thr Gln Val Pro Pro Gly Gly Lys Asp Gly Leu Arg Pro Asp Gly
                485                 490                 495

ACA GCA TGG CGA CAT GAA GGC AAC TAT CCG GGC TAC TCT TTC CCA GCC      1654
Thr Ala Trp Arg His Glu Gly Asn Tyr Pro Gly Tyr Ser Phe Pro Ala
            500                 505                 510

TTT AAA AAT GCC TCT CAG CTT ATT TAT TTA TTA CGC GAT ACA CCA TTT      1702
Phe Lys Asn Ala Ser Gln Leu Ile Tyr Leu Leu Arg Asp Thr Pro Phe
        515                 520                 525

TCA GTG GGT GAA AGT GGT TGG AAT AAC CTG AAA AAA GCG ATG GTT TCA      1750
Ser Val Gly Glu Ser Gly Trp Asn Asn Leu Lys Lys Ala Met Val Ser
    530                 535                 540

GCG TGG ATC TAC AGT AAT CCA GAA GTT GGA TTA CCG CTT GCA GGA AGA      1798
Ala Trp Ile Tyr Ser Asn Pro Glu Val Gly Leu Pro Leu Ala Gly Arg
545                 550                 555                 560

CAC CCT TTT AAC TCA CCT TCG TTA AAA TCA GTC GCT CAA GGC TAT TAC      1846
His Pro Phe Asn Ser Pro Ser Leu Lys Ser Val Ala Gln Gly Tyr Tyr
                565                 570                 575

TGG CTT GCC ATG TCT GCA AAA TCA TCG CCT GAT AAA ACA CTT GCA TCT      1894
Trp Leu Ala Met Ser Ala Lys Ser Ser Pro Asp Lys Thr Leu Ala Ser
            580                 585                 590

ATT TAT CTT GCG ATT AGT GAT AAA ACA CAA AAT GAA TCA ACT GCT ATT      1942
Ile Tyr Leu Ala Ile Ser Asp Lys Thr Gln Asn Glu Ser Thr Ala Ile
        595                 600                 605

TTT GGA GAA ACT ATT ACA CCA GCG TCT TTA CCT CAA GGT TTC TAT GCC      1990
Phe Gly Glu Thr Ile Thr Pro Ala Ser Leu Pro Gln Gly Phe Tyr Ala
    610                 615                 620

TTT AAT GGC GGT GCT TTT GGT ATT CAT CGT TGG CAA GAT AAA ATG GTG      2038
Phe Asn Gly Gly Ala Phe Gly Ile His Arg Trp Gln Asp Lys Met Val
625                 630                 635                 640

ACA CTG AAA GCT TAT AAC ACC AAT GTT TGG TCA TCT GAA ATT TAT AAC      2086
Thr Leu Lys Ala Tyr Asn Thr Asn Val Trp Ser Ser Glu Ile Tyr Asn
                645                 650                 655

AAA GAT AAC CGT TAT GGC CGT TAC CAA AGT CAT GGT GTC GCT CAA ATA      2134
Lys Asp Asn Arg Tyr Gly Arg Tyr Gln Ser His Gly Val Ala Gln Ile
            660                 665                 670

GTG AGT AAT GGC TCG CAG CTT TCA CAG GGC TAT CAG CAA GAA GGT TGG      2182
Val Ser Asn Gly Ser Gln Leu Ser Gln Gly Tyr Gln Gln Glu Gly Trp
        675                 680                 685

GAT TGG AAT AGA ATG CAA GGG GCA ACC ACT ATT CAC CTT CCT CTT AAA      2230
Asp Trp Asn Arg Met Gln Gly Ala Thr Thr Ile His Leu Pro Leu Lys
    690                 695                 700

GAC TTA GAC AGT CCT AAA CCT CAT ACC TTA ATG CAA CGT GGA GAG CGT      2278
Asp Leu Asp Ser Pro Lys Pro His Thr Leu Met Gln Arg Gly Glu Arg
705                 710                 715                 720

GGA TTT AGC GGA ACA TCA TCC CTT GAA GGT CAA TAT GGC ATG ATG GCA      2326
Gly Phe Ser Gly Thr Ser Ser Leu Glu Gly Gln Tyr Gly Met Met Ala
```

```
                    725                 730                 735

TTC GAT CTT ATT TAT CCC GCC AAT CTT GAG CGT TTT GAT CCT AAT TTC      2374
Phe Asp Leu Ile Tyr Pro Ala Asn Leu Glu Arg Phe Asp Pro Asn Phe
            740                 745                 750

ACT GCG AAA AAG AGT GTA TTA GCC GCT GAT AAT CAC TTA ATT TTT ATT      2422
Thr Ala Lys Lys Ser Val Leu Ala Ala Asp Asn His Leu Ile Phe Ile
        755                 760                 765

GGT AGC AAT ATA AAT AGT AGT GAT AAA AAT AAA AAT GTT GAA ACG ACC      2470
Gly Ser Asn Ile Asn Ser Ser Asp Lys Asn Lys Asn Val Glu Thr Thr
    770                 775                 780

TTA TTC CAA CAT GCC ATT ACT CCA ACA TTA AAT ACC CTT TGG ATT AAT      2518
Leu Phe Gln His Ala Ile Thr Pro Thr Leu Asn Thr Leu Trp Ile Asn
785                 790                 795                 800

GGA CAA AAG ATA GAA AAC ATG CCT TAT CAA ACA ACA CTT CAA CAA GGT      2566
Gly Gln Lys Ile Glu Asn Met Pro Tyr Gln Thr Thr Leu Gln Gln Gly
                805                 810                 815

GAT TGG TTA ATT GAT AGC AAT GGC AAT GGT TAC TTA ATT ACT CAA GCA      2614
Asp Trp Leu Ile Asp Ser Asn Gly Asn Gly Tyr Leu Ile Thr Gln Ala
            820                 825                 830

GAA AAA GTA AAT GTA AGT CGC CAA CAT CAG GTT TCA GCG GAA AAT AAA      2662
Glu Lys Val Asn Val Ser Arg Gln His Gln Val Ser Ala Glu Asn Lys
        835                 840                 845

AAT CGC CAA CCG ACA GAA GGA AAC TTT AGC TCG GCA TGG ATC GAT CAC      2710
Asn Arg Gln Pro Thr Glu Gly Asn Phe Ser Ser Ala Trp Ile Asp His
    850                 855                 860

AGC ACT CGC CCC AAA GAT GCC AGT TAT GAG TAT ATG GTC TTT TTA GAT      2758
Ser Thr Arg Pro Lys Asp Ala Ser Tyr Glu Tyr Met Val Phe Leu Asp
865                 870                 875                 880

GCG ACA CCT GAA AAA ATG GGA GAG ATG GCA CAA AAA TTC CGT GAA AAT      2806
Ala Thr Pro Glu Lys Met Gly Glu Met Ala Gln Lys Phe Arg Glu Asn
                885                 890                 895

AAT GGG TTA TAT CAG GTT CTT CGT AAG GAT AAA GAC GTT CAT ATT ATT      2854
Asn Gly Leu Tyr Gln Val Leu Arg Lys Asp Lys Asp Val His Ile Ile
            900                 905                 910

CTC GAT AAA CTC AGC AAT GTA ACG GGA TAT GCC TTT TAT CAG CCA GCA      2902
Leu Asp Lys Leu Ser Asn Val Thr Gly Tyr Ala Phe Tyr Gln Pro Ala
        915                 920                 925

TCA ATT GAA GAC AAA TGG ATC AAA AAG GTT AAT AAA CCT GCA ATT GTG      2950
Ser Ile Glu Asp Lys Trp Ile Lys Lys Val Asn Lys Pro Ala Ile Val
    930                 935                 940

ATG ACT CAT CGA CAA AAA GAC ACT CTT ATT GTC AGT GCA GTT ACA CCT      2998
Met Thr His Arg Gln Lys Asp Thr Leu Ile Val Ser Ala Val Thr Pro
945                 950                 955                 960

GAT TTA AAT ATG ACT CGC CAA AAA GCA GCA ACT CCT GTC ACC ATC AAT      3046
Asp Leu Asn Met Thr Arg Gln Lys Ala Ala Thr Pro Val Thr Ile Asn
                965                 970                 975
```

```
GTC ACG ATT AAT GGC AAA TGG CAA TCT GCT GAT AAA AAT AGT GAA GTG      3094
Val Thr Ile Asn Gly Lys Trp Gln Ser Ala Asp Lys Asn Ser Glu Val
            980                 985                 990

AAA TAT CAG GTT TCT GGT GAT AAC ACT GAA CTG ACG TTT ACG AGT TAC      3142
Lys Tyr Gln Val Ser Gly Asp Asn Thr Glu Leu Thr Phe Thr Ser Tyr
        995                1000                1005

TTT GGT ATT CCA CAA GAA ATC AAA CTC TCG CCA CTC CCT TGATTTAATC       3191
Phe Gly Ile Pro Gln Glu Ile Lys Leu Ser Pro Leu Pro
    1010                1015                1020

AAAAGAACGC TCTTGCGTTC CTTTTTTATT TGCAGGAAAT CTGATTATGC TAATAAAAAA    3251

CCCTTTAGCC CACGCGGTTA CATTAAGCCT CTGTTTATCA TTACCCGCAC AAGCATTACC    3311

CACTCTGTCT CATGAAGCTT TCGGCGATAT TTATCTTTTT GAAGGTGAAT TACCCAATAC    3371

CCTTACCACT TCAAATAATA ATCAATTATC GCTAAGCAAA CAGCATGCTA AAGATGGTGA    3431

ACAATCACTC AAATGGCAAT ATCAACCACA AGCAACATTA ACACTAAATA ATATTGTTAA    3491

TTACCAAGAT GATAAAAATA CAGCCACACC ACTCACTTTT ATGATGTGGA TTTATAATGA    3551

AAAACCTCAA TCTTCCCCAT TAACGTTAGC ATTTAAACAA AATAATAAAA TTGCACTAAG    3611

TTTTAATGCT GAACTTAATT TTACGGGGTG GCGAGGTATT GCTGTTCCTT TTCGTGATAT    3671

GCAAGGCTCT GCGACAGGTC AACTTGATCA ATTAGTGATC ACCGCTCCAA ACCAAGCCGG    3731

AACACTCTTT TTTGATCAAA TCATCATGAG TGTACCGTTA GACAATCGTT GGGCAGTACC    3791

TGACTATCAA ACACCTTACG TAAATAACGC AGTAAACACG ATGGTTAGTA AAAACTGGAG    3851

TGCATTATTG ATGTACGATC AGATGTTTCA AGCCCATTAC CCTACTTTAA ACTTCGATAC    3911

TGAATTTCGC GATGACCAAA CAGAAATGGC TTCGATTTAT CAGCGCTTTG AATATTATCA    3971

AGGAATTCC                                                           3980
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1021 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Pro Ile Phe Arg Phe Thr Ala Leu Ala Met Thr Leu Gly Leu Leu
  1               5                  10                  15

Ser Ala Pro Tyr Asn Ala Met Ala Ala Thr Ser Asn Pro Ala Phe Asp
            20                  25                  30

Pro Lys Asn Leu Met Gln Ser Glu Ile Tyr His Phe Ala Gln Asn Asn
        35                  40                  45

Pro Leu Ala Asp Phe Ser Ser Asp Lys Asn Ser Ile Leu Thr Leu Ser
```

```
     50                  55                  60

Asp Lys Arg Ser Ile Met Gly Asn Gln Ser Leu Leu Trp Lys Trp Lys
 65                  70                  75                  80

Gly Gly Ser Ser Phe Thr Leu His Lys Lys Leu Ile Val Pro Thr Asp
                 85                  90                  95

Lys Glu Ala Ser Lys Ala Trp Gly Arg Ser Ser Thr Pro Val Phe Ser
            100                 105                 110

Phe Trp Leu Tyr Asn Glu Lys Pro Ile Asp Gly Tyr Leu Thr Ile Asp
        115                 120                 125

Phe Gly Glu Lys Leu Ile Ser Thr Ser Glu Ala Gln Ala Gly Phe Lys
    130                 135                 140

Val Lys Leu Asp Phe Thr Gly Trp Arg Ala Val Gly Val Ser Leu Asn
145                 150                 155                 160

Asn Asp Leu Glu Asn Arg Glu Met Thr Leu Asn Ala Thr Asn Thr Ser
                165                 170                 175

Ser Asp Gly Thr Gln Asp Ser Ile Gly Arg Ser Leu Gly Ala Lys Val
            180                 185                 190

Asp Ser Ile Arg Phe Lys Ala Pro Ser Asn Val Ser Gln Gly Glu Ile
        195                 200                 205

Tyr Ile Asp Arg Ile Met Phe Ser Val Asp Asp Ala Arg Tyr Gln Trp
    210                 215                 220

Ser Asp Tyr Gln Val Lys Thr Arg Leu Ser Glu Pro Glu Ile Gln Phe
225                 230                 235                 240

His Asn Val Lys Pro Gln Leu Pro Val Thr Pro Glu Asn Leu Ala Ala
                245                 250                 255

Ile Asp Leu Ile Arg Gln Arg Leu Ile Asn Glu Phe Val Gly Gly Glu
            260                 265                 270

Lys Glu Thr Asn Leu Ala Leu Glu Glu Asn Ile Ser Lys Leu Lys Ser
        275                 280                 285

Asp Phe Asp Ala Leu Asn Ile His Thr Leu Ala Asn Gly Gly Thr Gln
    290                 295                 300

Gly Arg His Leu Ile Thr Asp Lys Gln Ile Ile Ile Tyr Gln Pro Glu
305                 310                 315                 320

Asn Leu Asn Ser Gln Asp Lys Gln Leu Phe Asp Asn Tyr Val Ile Leu
                325                 330                 335

Gly Asn Tyr Thr Thr Leu Met Phe Asn Ile Ser Arg Ala Tyr Val Leu
            340                 345                 350

Glu Lys Asp Pro Thr Gln Lys Ala Gln Leu Lys Gln Met Tyr Leu Leu
        355                 360                 365

Met Thr Lys His Leu Leu Asp Gln Gly Phe Val Lys Gly Ser Ala Leu
    370                 375                 380
```

```
Val Thr Thr His His Trp Gly Tyr Ser Ser Arg Trp Trp Tyr Ile Ser
385                 390                 395                 400

Thr Leu Leu Met Ser Asp Ala Leu Lys Glu Ala Asn Leu Gln Thr Gln
                405                 410                 415

Val Tyr Asp Ser Leu Leu Trp Tyr Ser Arg Glu Phe Lys Ser Ser Phe
            420                 425                 430

Asp Met Lys Val Ser Ala Asp Ser Ser Asp Leu Asp Tyr Phe Asn Thr
        435                 440                 445

Leu Ser Arg Gln His Leu Ala Leu Leu Leu Leu Glu Pro Asp Asp Gln
    450                 455                 460

Lys Arg Ile Asn Leu Val Asn Thr Phe Ser His Tyr Ile Thr Gly Ala
465                 470                 475                 480

Leu Thr Gln Val Pro Pro Gly Gly Lys Asp Gly Leu Arg Pro Asp Gly
                485                 490                 495

Thr Ala Trp Arg His Glu Gly Asn Tyr Pro Gly Tyr Ser Phe Pro Ala
            500                 505                 510

Phe Lys Asn Ala Ser Gln Leu Ile Tyr Leu Leu Arg Asp Thr Pro Phe
        515                 520                 525

Ser Val Gly Glu Ser Gly Trp Asn Asn Leu Lys Lys Ala Met Val Ser
    530                 535                 540

Ala Trp Ile Tyr Ser Asn Pro Glu Val Gly Leu Pro Leu Ala Gly Arg
545                 550                 555                 560

His Pro Phe Asn Ser Pro Ser Leu Lys Ser Val Ala Gln Gly Tyr Tyr
                565                 570                 575

Trp Leu Ala Met Ser Ala Lys Ser Ser Pro Asp Lys Thr Leu Ala Ser
            580                 585                 590

Ile Tyr Leu Ala Ile Ser Asp Lys Thr Gln Asn Glu Ser Thr Ala Ile
        595                 600                 605

Phe Gly Glu Thr Ile Thr Pro Ala Ser Leu Pro Gln Gly Phe Tyr Ala
    610                 615                 620

Phe Asn Gly Gly Ala Phe Gly Ile His Arg Trp Gln Asp Lys Met Val
625                 630                 635                 640

Thr Leu Lys Ala Tyr Asn Thr Asn Val Trp Ser Ser Glu Ile Tyr Asn
                645                 650                 655

Lys Asp Asn Arg Tyr Gly Arg Tyr Gln Ser His Gly Val Ala Gln Ile
            660                 665                 670

Val Ser Asn Gly Ser Gln Leu Ser Gln Gly Tyr Gln Gln Glu Gly Trp
        675                 680                 685

Asp Trp Asn Arg Met Gln Gly Ala Thr Thr Ile His Leu Pro Leu Lys
    690                 695                 700

Asp Leu Asp Ser Pro Lys Pro His Thr Leu Met Gln Arg Gly Glu Arg
705                 710                 715                 720
```

Gly Phe Ser Gly Thr Ser Ser Leu Glu Gly Gln Tyr Gly Met Met Ala
725 730 735

Phe Asp Leu Ile Tyr Pro Ala Asn Leu Glu Arg Phe Asp Pro Asn Phe
740 745 750

Thr Ala Lys Lys Ser Val Leu Ala Ala Asp Asn His Leu Ile Phe Ile
755 760 765

Gly Ser Asn Ile Asn Ser Ser Asp Lys Asn Lys Asn Val Glu Thr Thr
770 775 780

Leu Phe Gln His Ala Ile Thr Pro Thr Leu Asn Thr Leu Trp Ile Asn
785 790 795 800

Gly Gln Lys Ile Glu Asn Met Pro Tyr Gln Thr Thr Leu Gln Gln Gly
805 810 815

Asp Trp Leu Ile Asp Ser Asn Gly Asn Gly Tyr Leu Ile Thr Gln Ala
820 825 830

Glu Lys Val Asn Val Ser Arg Gln His Gln Val Ser Ala Glu Asn Lys
835 840 845

Asn Arg Gln Pro Thr Glu Gly Asn Phe Ser Ser Ala Trp Ile Asp His
850 855 860

Ser Thr Arg Pro Lys Asp Ala Ser Tyr Glu Tyr Met Val Phe Leu Asp
865 870 875 880

Ala Thr Pro Glu Lys Met Gly Glu Met Ala Gln Lys Phe Arg Glu Asn
885 890 895

Asn Gly Leu Tyr Gln Val Leu Arg Lys Asp Lys Asp Val His Ile Ile
900 905 910

Leu Asp Lys Leu Ser Asn Val Thr Gly Tyr Ala Phe Tyr Gln Pro Ala
915 920 925

Ser Ile Glu Asp Lys Trp Ile Lys Lys Val Asn Lys Pro Ala Ile Val
930 935 940

Met Thr His Arg Gln Lys Asp Thr Leu Ile Val Ser Ala Val Thr Pro
945 950 955 960

Asp Leu Asn Met Thr Arg Gln Lys Ala Ala Thr Pro Val Thr Ile Asn
965 970 975

Val Thr Ile Asn Gly Lys Trp Gln Ser Ala Asp Lys Asn Ser Glu Val
980 985 990

Lys Tyr Gln Val Ser Gly Asp Asn Thr Glu Leu Thr Phe Thr Ser Tyr
995 1000 1005

Phe Gly Ile Pro Gln Glu Ile Lys Leu Ser Pro Leu Pro
1010 1015 1020

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3980 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGAATTCCAT CACTCAATCA TTAAATTTAG GCACAACGAT GGGCTATCAG CGTTATGACA 60

AATTTAATGA AGGACGCATT GGTTTCACTG TTAGCCAGCG TTTCTAAGGA GAAAACATAT 120

GCCGATATTT CGTTTTACTG CACTTGCAAT GACATTGGGG CTATTATCAG CGCCTTATAA 180

CGCGATGGCA GCCACCAGCA ATCCTGCATT TGATCCTAAA AATCTGATGC AGTCAGAAAT 240

TTACCATTTT GCACAAAATA ACCCATTAGC AGACTTCTCA TCAGATAAAA ACTCAATACT 300

AACGTTATCT GATAAACGTA GCATTATGGG AAACCAATCT CTTTTATGGA AATGGAAAGG 360

TGGTAGTAGC TTTACTTTAC ATAAAAAACT GATTGTCCCC ACCGATAAAG AAGCATCTAA 420

AGCATGGGGA CGCTCATCTA CCCCCGTTTT CTCATTTTGG CTTTACAATG AAAAACCGAT 480

TGATGGTTAT CTTACTATCG ATTTCGGAGA AAAACTCATT TCAACCAGTG AGGCTCAGGC 540

AGGCTTTAAA GTAAAATTAG ATTTCACTGG CTGGCGTGCT GTGGGAGTCT CTTTAAATAA 600

CGATCTTGAA AATCGAGAGA TGACCTTAAA TGCAACCAAT ACCTCCTCTG ATGGTACTCA 660

AGACAGCATT GGGCGTTCTT TAGGTGCTAA AGTCGATAGT ATTCGTTTTA AAGCGCCTTC 720

TAATGTGAGT CAGGGTGAAA TCTATATCGA CCGTATTATG TTTTCTGTCG ATGATGCTCG 780

CTACCAATGG TCTGATTATC AAGTAAAAAC TCGCTTATCA GAACCTGAAA TTCAATTTCA 840

CAACGTAAAG CCACAACTAC CTGTAACACC TGAAAATTTA GCGGCCATTG ATCTTATTCG 900

CCAACGTCTA ATTAATGAAT TTGTCGGAGG TGAAAAAGAG ACAAACCTCG CATTAGAAGA 960

GAATATCAGC AAATTAAAAA GTGATTTCGA TGCTCTTAAT ATTCACACTT TAGCAAATGG 1020

TGGAACGCAA GGCAGACATC TGATCACTGA TAAACAAATC ATTATTTATC AACCAGAGAA 1080

TCTTAACTCC CAAGATAAAC AACTATTTGA TAATTATGTT ATTTTAGGTA ATTACACGAC 1140

ATTAATGTTT AATATTAGCC GTGCTTATGT GCTGGAAAAA GATCCCACAC AAAAGGCGCA 1200

ACTAAAGCAG ATGTACTTAT TAATGACAAA GCATTTATTA GATCAAGGCT TTGTTAAAGG 1260

GAGTGCTTTA GTGACAACCC ATCACTGGGG ATACAGTTCT CGTTGGTGGT ATATTTCCAC 1320

GTTATTAATG TCTGATGCAC TAAAAGAAGC GAACCTACAA ACTCAAGTTT ATGATTCATT 1380

ACTGTGGTAT TCACGTGAGT TTAAAAGTAG TTTTGATATG AAAGTAAGTG CTGATAGCTC 1440

TGATCTAGAT TATTTCAATA CCTTATCTCG CCAACATTTA GCCTTATTAT TACTAGAGCC 1500

TGATGATCAA AAGCGTATCA ACTTAGTTAA TACTTTCAGC CATTATATCA CTGGCGCATT 1560

AACGCAAGTG CCACCGGGTG GTAAAGATGG TTTACGCCCT GATGGTACAG CATGGCGACA 1620

TGAAGGCAAC TATCCGGGCT ACTCTTTCCC AGCCTTTAAA AATGCCTCTC AGCTTATTTA 1680

TTTATTACGC GATACACCAT TTTCAGTGGG TGAAAGTGGT TGGAATAACC TGAAAAAAGC 1740

GATGGTTTCA GCGTGGATCT ACAGTAATCC AGAAGTTGGA TTACCGCTTG CAGGAAGACA 1800

CCCTTTTAAC TCACCTTCGT TAAAATCAGT CGCTCAAGGC TATTACTGGC TTGCCATGTC 1860

TGCAAAATCA TCGCCTGATA AAACACTTGC ATCTATTTAT CTTGCGATTA GTGATAAAAC 1920

ACAAAATGAA TCAACTGCTA TTTTTGGAGA AACTATTACA CCAGCGTCTT TACCTCAAGG 1980

TTTCTATGCC TTTAATGGCG GTGCTTTTGG TATTCATCGT TGGCAAGATA AAATGGTGAC 2040

ACTGAAAGCT TATAACACCA ATGTTTGGTC ATCTGAAATT TATAACAAAG ATAACCGTTA 2100

TGGCCGTTAC CAAAGTCATG GTGTCGCTCA AATAGTGAGT AATGGCTCGC AGCTTTCACA 2160

GGGCTATCAG CAAGAAGGTT GGGATTGGAA TAGAATGCAA GGGGCAACCA CTATTCACCT 2220

TCCTCTTAAA GACTTAGACA GTCCTAAACC TCATACCTTA ATGCAACGTG GAGAGCGTGG 2280

ATTTAGCGGA ACATCATCCC TTGAAGGTCA ATATGGCATG ATGGCATTCG ATCTTATTTA 2340

TCCCGCCAAT CTTGAGCGTT TTGATCCTAA TTTCACTGCG AAAAAGAGTG TATTAGCCGC 2400

TGATAATCAC TTAATTTTTA TTGGTAGCAA TATAAATAGT AGTGATAAAA ATAAAAATGT 2460

TGAAACGACC TTATTCCAAC ATGCCATTAC TCCAACATTA AATACCCTTT GGATTAATGG 2520

ACAAAAGATA GAAAACATGC CTTATCAAAC AACACTTCAA CAAGGTGATT GGTTAATTGA 2580

TAGCAATGGC AATGGTTACT TAATTACTCA AGCAGAAAAA GTAAATGTAA GTCGCCAACA 2640

TCAGGTTTCA GCGGAAAATA AAAATCGCCA ACCGACAGAA GGAAACTTTA GCTCGGCATG 2700

GATCGATCAC AGCACTCGCC CCAAAGATGC CAGTTATGAG TATATGGTCT TTTTAGATGC 2760

GACACCTGAA AAAATGGGAG AGATGGCACA AAAATTCCGT GAAAATAATG GGTTATATCA 2820

GGTTCTTCGT AAGGATAAAG ACGTTCATAT TATTCTCGAT AAACTCAGCA ATGTAACGGG 2880

ATATGCCTTT TATCAGCCAG CATCAATTGA AGACAAATGG ATCAAAAAGG TTAATAAACC 2940

TGCAATTGTG ATGACTCATC GACAAAAAGA CACTCTTATT GTCAGTGCAG TTACACCTGA 3000

TTTAAATATG ACTCGCCAAA AAGCAGCAAC TCCTGTCACC ATCAATGTCA CGATTAATGG 3060

CAAATGGCAA TCTGCTGATA AAAATAGTGA AGTGAAATAT CAGGTTTCTG GTGATAACAC 3120

TGAACTGACG TTTACGAGTT ACTTTGGTAT TCCACAAGAA ATCAAACTCT CGCCACTCCC 3180

TTGATTTAAT CAAAAGAACG CTCTTGCGTT CCTTTTTTAT TTGCAGGAAA TCTGATTATG 3240

CTAATAAAAA ACCCTTTAGC CCACGCGGTT ACATTAAGCC TCTGTTTATC ATTACCCGCA 3300

CAAGCATTAC CCACTCTGTC TCATGAAGCT TTCGGCGATA TTTATCTTTT TGAAGGTGAA 3360

```
TTACCCAATA CCCTTACCAC TTCAAATAAT AATCAATTAT CGCTAAGCAA ACAGCATGCT    3420

AAAGATGGTG AACAATCACT CAAATGGCAA TATCAACCAC AAGCAACATT AACACTAAAT    3480

AATATTGTTA ATTACCAAGA TGATAAAAAT ACAGCCACAC CACTCACTTT TATGATGTGG    3540

ATTTATAATG AAAAACCTCA ATCTTCCCCA TTAACGTTAG CATTTAAACA AAATAATAAA    3600

ATTGCACTAA GTTTTAATGC TGAACTTAAT TTTACGGGGT GGCGAGGTAT TGCTGTTCCT    3660

TTTCGTGATA TGCAAGGCTC TGCGACAGGT CAACTTGATC AATTAGTGAT CACCGCTCCA    3720

AACCAAGCCG GAACACTCTT TTTTGATCAA ATCATCATGA GTGTACCGTT AGACAATCGT    3780

TGGGCAGTAC CTGACTATCA AACACCTTAC GTAAATAACG CAGTAAACAC GATGGTTAGT    3840

AAAAACTGGA GTGCATTATT GATGTACGAT CAGATGTTTC AAGCCCATTA CCCTACTTTA    3900

AACTTCGATA CTGAATTTCG CGATGACCAA ACAGAAATGG CTTCGATTTA TCAGCGCTTT    3960

GAATATTATC AAGGAATTCC                                                3980
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3980 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 188..3181

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GGAATTCCAT CACTCAATCA TTAAATTTAG GCACAACGAT GGGCTATCAG CGTTATGACA      60

AATTTAATGA AGGACGCATT GGTTTCACTG TTAGCCAGCG TTTCTAAGGA GAAAAATAAT     120

GCCGATATTT CGTTTTACTG CACTTGCAAT GACATTGGGG CTATTATCAG CGCCTTATAA     180

CGCGGAT ATG GCC ACC AGC AAT CCT GCA TTT GAT CCT AAA AAT CTG ATG        229
        Met Ala Thr Ser Asn Pro Ala Phe Asp Pro Lys Asn Leu Met
          1               5                  10

CAG TCA GAA ATT TAC CAT TTT GCA CAA AAT AAC CCA TTA GCA GAC TTC       277
Gln Ser Glu Ile Tyr His Phe Ala Gln Asn Asn Pro Leu Ala Asp Phe
 15                  20                  25                  30

TCA TCA GAT AAA AAC TCA ATA CTA ACG TTA TCT GAT AAA CGT AGC ATT       325
Ser Ser Asp Lys Asn Ser Ile Leu Thr Leu Ser Asp Lys Arg Ser Ile
                35                  40                  45
```

```
ATG GGA AAC CAA TCT CTT TTA TGG AAA TGG AAA GGT GGT AGT AGC TTT          373
Met Gly Asn Gln Ser Leu Leu Trp Lys Trp Lys Gly Gly Ser Ser Phe
                50                  55                  60

ACT TTA CAT AAA AAA CTG ATT GTC CCC ACC GAT AAA GAA GCA TCT AAA          421
Thr Leu His Lys Lys Leu Ile Val Pro Thr Asp Lys Glu Ala Ser Lys
        65                  70                  75

GCA TGG GGA CGC TCA TCT ACC CCC GTT TTC TCA TTT TGG CTT TAC AAT          469
Ala Trp Gly Arg Ser Ser Thr Pro Val Phe Ser Phe Trp Leu Tyr Asn
     80                  85                  90

GAA AAA CCG ATT GAT GGT TAT CTT ACT ATC GAT TTC GGA GAA AAA CTC          517
Glu Lys Pro Ile Asp Gly Tyr Leu Thr Ile Asp Phe Gly Glu Lys Leu
 95                 100                 105                 110

ATT TCA ACC AGT GAG GCT CAG GCA GGC TTT AAA GTA AAA TTA GAT TTC          565
Ile Ser Thr Ser Glu Ala Gln Ala Gly Phe Lys Val Lys Leu Asp Phe
                115                 120                 125

ACT GGC TGG CGT GCT GTG GGA GTC TCT TTA AAT AAC GAT CTT GAA AAT          613
Thr Gly Trp Arg Ala Val Gly Val Ser Leu Asn Asn Asp Leu Glu Asn
            130                 135                 140

CGA GAG ATG ACC TTA AAT GCA ACC AAT ACC TCC TCT GAT GGT ACT CAA          661
Arg Glu Met Thr Leu Asn Ala Thr Asn Thr Ser Ser Asp Gly Thr Gln
        145                 150                 155

GAC AGC ATT GGG CGT TCT TTA GGT GCT AAA GTC GAT AGT ATT CGT TTT          709
Asp Ser Ile Gly Arg Ser Leu Gly Ala Lys Val Asp Ser Ile Arg Phe
    160                 165                 170

AAA GCG CCT TCT AAT GTG AGT CAG GGT GAA ATC TAT ATC GAC CGT ATT          757
Lys Ala Pro Ser Asn Val Ser Gln Gly Glu Ile Tyr Ile Asp Arg Ile
175                 180                 185                 190

ATG TTT TCT GTC GAT GAT GCT CGC TAC CAA TGG TCT GAT TAT CAA GTA          805
Met Phe Ser Val Asp Asp Ala Arg Tyr Gln Trp Ser Asp Tyr Gln Val
                195                 200                 205

AAA ACT CGC TTA TCA GAA CCT GAA ATT CAA TTT CAC AAC GTA AAG CCA          853
Lys Thr Arg Leu Ser Glu Pro Glu Ile Gln Phe His Asn Val Lys Pro
            210                 215                 220

CAA CTA CCT GTA ACA CCT GAA AAT TTA GCG GCC ATT GAT CTT ATT CGC          901
Gln Leu Pro Val Thr Pro Glu Asn Leu Ala Ala Ile Asp Leu Ile Arg
        225                 230                 235

CAA CGT CTA ATT AAT GAA TTT GTC GGA GGT GAA AAA GAG ACA AAC CTC          949
Gln Arg Leu Ile Asn Glu Phe Val Gly Gly Glu Lys Glu Thr Asn Leu
    240                 245                 250

GCA TTA GAA GAG AAT ATC AGC AAA TTA AAA AGT GAT TTC GAT GCT CTT          997
Ala Leu Glu Glu Asn Ile Ser Lys Leu Lys Ser Asp Phe Asp Ala Leu
255                 260                 265                 270

AAT ATT CAC ACT TTA GCA AAT GGT GGA ACG CAA GGC AGA CAT CTG ATC         1045
Asn Ile His Thr Leu Ala Asn Gly Gly Thr Gln Gly Arg His Leu Ile
                275                 280                 285

ACT GAT AAA CAA ATC ATT ATT TAT CAA CCA GAG AAT CTT AAC TCC CAA         1093
Thr Asp Lys Gln Ile Ile Ile Tyr Gln Pro Glu Asn Leu Asn Ser Gln
```

```
                290                 295                 300

GAT AAA CAA CTA TTT GAT AAT TAT GTT ATT TTA GGT AAT TAC ACG ACA      1141
Asp Lys Gln Leu Phe Asp Asn Tyr Val Ile Leu Gly Asn Tyr Thr Thr
        305                 310                 315

TTA ATG TTT AAT ATT AGC CGT GCT TAT GTG CTG GAA AAA GAT CCC ACA      1189
Leu Met Phe Asn Ile Ser Arg Ala Tyr Val Leu Glu Lys Asp Pro Thr
    320                 325                 330

CAA AAG GCG CAA CTA AAG CAG ATG TAC TTA TTA ATG ACA AAG CAT TTA      1237
Gln Lys Ala Gln Leu Lys Gln Met Tyr Leu Leu Met Thr Lys His Leu
335                 340                 345                 350

TTA GAT CAA GGC TTT GTT AAA GGG AGT GCT TTA GTG ACA ACC CAT CAC      1285
Leu Asp Gln Gly Phe Val Lys Gly Ser Ala Leu Val Thr Thr His His
                355                 360                 365

TGG GGA TAC AGT TCT CGT TGG TGG TAT ATT TCC ACG TTA TTA ATG TCT      1333
Trp Gly Tyr Ser Ser Arg Trp Trp Tyr Ile Ser Thr Leu Leu Met Ser
            370                 375                 380

GAT GCA CTA AAA GAA GCG AAC CTA CAA ACT CAA GTT TAT GAT TCA TTA      1381
Asp Ala Leu Lys Glu Ala Asn Leu Gln Thr Gln Val Tyr Asp Ser Leu
        385                 390                 395

CTG TGG TAT TCA CGT GAG TTT AAA AGT AGT TTT GAT ATG AAA GTA AGT      1429
Leu Trp Tyr Ser Arg Glu Phe Lys Ser Ser Phe Asp Met Lys Val Ser
    400                 405                 410

GCT GAT AGC TCT GAT CTA GAT TAT TTC AAT ACC TTA TCT CGC CAA CAT      1477
Ala Asp Ser Ser Asp Leu Asp Tyr Phe Asn Thr Leu Ser Arg Gln His
415                 420                 425                 430

TTA GCC TTA TTA TTA CTA GAG CCT GAT GAT CAA AAG CGT ATC AAC TTA      1525
Leu Ala Leu Leu Leu Leu Glu Pro Asp Asp Gln Lys Arg Ile Asn Leu
                435                 440                 445

GTT AAT ACT TTC AGC CAT TAT ATC ACT GGC GCA TTA ACG CAA GTG CCA      1573
Val Asn Thr Phe Ser His Tyr Ile Thr Gly Ala Leu Thr Gln Val Pro
            450                 455                 460

CCG GGT GGT AAA GAT GGT TTA CGC CCT GAT GGT ACA GCA TGG CGA CAT      1621
Pro Gly Gly Lys Asp Gly Leu Arg Pro Asp Gly Thr Ala Trp Arg His
        465                 470                 475

GAA GGC AAC TAT CCG GGC TAC TCT TTC CCA GCC TTT AAA AAT GCC TCT      1669
Glu Gly Asn Tyr Pro Gly Tyr Ser Phe Pro Ala Phe Lys Asn Ala Ser
    480                 485                 490

CAG CTT ATT TAT TTA TTA CGC GAT ACA CCA TTT TCA GTG GGT GAA AGT      1717
Gln Leu Ile Tyr Leu Leu Arg Asp Thr Pro Phe Ser Val Gly Glu Ser
495                 500                 505                 510

GGT TGG AAT AAC CTG AAA AAA GCG ATG GTT TCA GCG TGG ATC TAC AGT      1765
Gly Trp Asn Asn Leu Lys Lys Ala Met Val Ser Ala Trp Ile Tyr Ser
                515                 520                 525

AAT CCA GAA GTT GGA TTA CCG CTT GCA GGA AGA CAC CCT TTT AAC TCA      1813
Asn Pro Glu Val Gly Leu Pro Leu Ala Gly Arg His Pro Phe Asn Ser
            530                 535                 540
```

```
CCT TCG TTA AAA TCA GTC GCT CAA GGC TAT TAC TGG CTT GCC ATG TCT      1861
Pro Ser Leu Lys Ser Val Ala Gln Gly Tyr Tyr Trp Leu Ala Met Ser
        545                 550                 555

GCA AAA TCA TCG CCT GAT AAA ACA CTT GCA TCT ATT TAT CTT GCG ATT      1909
Ala Lys Ser Ser Pro Asp Lys Thr Leu Ala Ser Ile Tyr Leu Ala Ile
    560                 565                 570

AGT GAT AAA ACA CAA AAT GAA TCA ACT GCT ATT TTT GGA GAA ACT ATT      1957
Ser Asp Lys Thr Gln Asn Glu Ser Thr Ala Ile Phe Gly Glu Thr Ile
575                 580                 585                 590

ACA CCA GCG TCT TTA CCT CAA GGT TTC TAT GCC TTT AAT GGC GGT GCT      2005
Thr Pro Ala Ser Leu Pro Gln Gly Phe Tyr Ala Phe Asn Gly Gly Ala
                595                 600                 605

TTT GGT ATT CAT CGT TGG CAA GAT AAA ATG GTG ACA CTG AAA GCT TAT      2053
Phe Gly Ile His Arg Trp Gln Asp Lys Met Val Thr Leu Lys Ala Tyr
            610                 615                 620

AAC ACC AAT GTT TGG TCA TCT GAA ATT TAT AAC AAA GAT AAC CGT TAT      2101
Asn Thr Asn Val Trp Ser Ser Glu Ile Tyr Asn Lys Asp Asn Arg Tyr
        625                 630                 635

GGC CGT TAC CAA AGT CAT GGT GTC GCT CAA ATA GTG AGT AAT GGC TCG      2149
Gly Arg Tyr Gln Ser His Gly Val Ala Gln Ile Val Ser Asn Gly Ser
    640                 645                 650

CAG CTT TCA CAG GGC TAT CAG CAA GAA GGT TGG GAT TGG AAT AGA ATG      2197
Gln Leu Ser Gln Gly Tyr Gln Gln Glu Gly Trp Asp Trp Asn Arg Met
655                 660                 665                 670

CAA GGG GCA ACC ACT ATT CAC CTT CCT CTT AAA GAC TTA GAC AGT CCT      2245
Gln Gly Ala Thr Thr Ile His Leu Pro Leu Lys Asp Leu Asp Ser Pro
                675                 680                 685

AAA CCT CAT ACC TTA ATG CAA CGT GGA GAG CGT GGA TTT AGC GGA ACA      2293
Lys Pro His Thr Leu Met Gln Arg Gly Glu Arg Gly Phe Ser Gly Thr
            690                 695                 700

TCA TCC CTT GAA GGT CAA TAT GGC ATG ATG GCA TTC GAT CTT ATT TAT      2341
Ser Ser Leu Glu Gly Gln Tyr Gly Met Met Ala Phe Asp Leu Ile Tyr
        705                 710                 715

CCC GCC AAT CTT GAG CGT TTT GAT CCT AAT TTC ACT GCG AAA AAG AGT      2389
Pro Ala Asn Leu Glu Arg Phe Asp Pro Asn Phe Thr Ala Lys Lys Ser
    720                 725                 730

GTA TTA GCC GCT GAT AAT CAC TTA ATT TTT ATT GGT AGC AAT ATA AAT      2437
Val Leu Ala Ala Asp Asn His Leu Ile Phe Ile Gly Ser Asn Ile Asn
735                 740                 745                 750

AGT AGT GAT AAA AAT AAA AAT GTT GAA ACG ACC TTA TTC CAA CAT GCC      2485
Ser Ser Asp Lys Asn Lys Asn Val Glu Thr Thr Leu Phe Gln His Ala
                755                 760                 765

ATT ACT CCA ACA TTA AAT ACC CTT TGG ATT AAT GGA CAA AAG ATA GAA      2533
Ile Thr Pro Thr Leu Asn Thr Leu Trp Ile Asn Gly Gln Lys Ile Glu
            770                 775                 780

AAC ATG CCT TAT CAA ACA ACA CTT CAA CAA GGT GAT TGG TTA ATT GAT      2581
Asn Met Pro Tyr Gln Thr Thr Leu Gln Gln Gly Asp Trp Leu Ile Asp
```

```
        785                 790                 795

AGC AAT GGC AAT GGT TAC TTA ATT ACT CAA GCA GAA AAA GTA AAT GTA      2629
Ser Asn Gly Asn Gly Tyr Leu Ile Thr Gln Ala Glu Lys Val Asn Val
    800                 805                 810

AGT CGC CAA CAT CAG GTT TCA GCG GAA AAT AAA AAT CGC CAA CCG ACA      2677
Ser Arg Gln His Gln Val Ser Ala Glu Asn Lys Asn Arg Gln Pro Thr
815                 820                 825                 830

GAA GGA AAC TTT AGC TCG GCA TGG ATC GAT CAC AGC ACT CGC CCC AAA      2725
Glu Gly Asn Phe Ser Ser Ala Trp Ile Asp His Ser Thr Arg Pro Lys
                835                 840                 845

GAT GCC AGT TAT GAG TAT ATG GTC TTT TTA GAT GCG ACA CCT GAA AAA      2773
Asp Ala Ser Tyr Glu Tyr Met Val Phe Leu Asp Ala Thr Pro Glu Lys
            850                 855                 860

ATG GGA GAG ATG GCA CAA AAA TTC CGT GAA AAT AAT GGG TTA TAT CAG      2821
Met Gly Glu Met Ala Gln Lys Phe Arg Glu Asn Asn Gly Leu Tyr Gln
        865                 870                 875

GTT CTT CGT AAG GAT AAA GAC GTT CAT ATT ATT CTC GAT AAA CTC AGC      2869
Val Leu Arg Lys Asp Lys Asp Val His Ile Ile Leu Asp Lys Leu Ser
    880                 885                 890

AAT GTA ACG GGA TAT GCC TTT TAT CAG CCA GCA TCA ATT GAA GAC AAA      2917
Asn Val Thr Gly Tyr Ala Phe Tyr Gln Pro Ala Ser Ile Glu Asp Lys
895                 900                 905                 910

TGG ATC AAA AAG GTT AAT AAA CCT GCA ATT GTG ATG ACT CAT CGA CAA      2965
Trp Ile Lys Lys Val Asn Lys Pro Ala Ile Val Met Thr His Arg Gln
                915                 920                 925

AAA GAC ACT CTT ATT GTC AGT GCA GTT ACA CCT GAT TTA AAT ATG ACT      3013
Lys Asp Thr Leu Ile Val Ser Ala Val Thr Pro Asp Leu Asn Met Thr
            930                 935                 940

CGC CAA AAA GCA GCA ACT CCT GTC ACC ATC AAT GTC ACG ATT AAT GGC      3061
Arg Gln Lys Ala Ala Thr Pro Val Thr Ile Asn Val Thr Ile Asn Gly
        945                 950                 955

AAA TGG CAA TCT GCT GAT AAA AAT AGT GAA GTG AAA TAT CAG GTT TCT      3109
Lys Trp Gln Ser Ala Asp Lys Asn Ser Glu Val Lys Tyr Gln Val Ser
    960                 965                 970

GGT GAT AAC ACT GAA CTG ACG TTT ACG AGT TAC TTT GGT ATT CCA CAA      3157
Gly Asp Asn Thr Glu Leu Thr Phe Thr Ser Tyr Phe Gly Ile Pro Gln
975                 980                 985                 990

GAA ATC AAA CTC TCG CCA CTC CCT TGATTTAATC AAAAGAACGC TCTTGCGTTC      3211
Glu Ile Lys Leu Ser Pro Leu Pro
                995

CTTTTTTATT TGCAGGAAAT CTGATTATGC TAATAAAAAA CCCTTTAGCC CACGCGGTTA     3271

CATTAAGCCT CTGTTTATCA TTACCCGCAC AAGCATTACC CACTCTGTCT CATGAAGCTT     3331

TCGGCGATAT TTATCTTTTT GAAGGTGAAT TACCCAATAC CCTTACCACT TCAAATAATA     3391

ATCAATTATC GCTAAGCAAA CAGCATGCTA AAGATGGTGA ACAATCACTC AAATGGCAAT     3451
```

ATCAACCACA AGCAACATTA ACACTAAATA ATATTGTTAA TTACCAAGAT GATAAAAATA 3511

CAGCCACACC ACTCACTTTT ATGATGTGGA TTTATAATGA AAAACCTCAA TCTTCCCCAT 3571

TAACGTTAGC ATTTAAACAA AATAATAAAA TTGCACTAAG TTTTAATGCT GAACTTAATT 3631

TTACGGGGTG GCGAGGTATT GCTGTTCCTT TTCGTGATAT GCAAGGCTCT GCGACAGGTC 3691

AACTTGATCA ATTAGTGATC ACCGCTCCAA ACCAAGCCGG AACACTCTTT TTTGATCAAA 3751

TCATCATGAG TGTACCGTTA GACAATCGTT GGGCAGTACC TGACTATCAA ACACCTTACG 3811

TAAATAACGC AGTAAACACG ATGGTTAGTA AAAACTGGAG TGCATTATTG ATGTACGATC 3871

AGATGTTTCA AGCCCATTAC CCTACTTTAA ACTTCGATAC TGAATTTCGC GATGACCAAA 3931

CAGAAATGGC TTCGATTTAT CAGCGCTTTG AATATTATCA AGGAATTCC 3980

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 998 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Ala Thr Ser Asn Pro Ala Phe Asp Pro Lys Asn Leu Met Gln Ser
  1               5                  10                  15

Glu Ile Tyr His Phe Ala Gln Asn Asn Pro Leu Ala Asp Phe Ser Ser
             20                  25                  30

Asp Lys Asn Ser Ile Leu Thr Leu Ser Asp Lys Arg Ser Ile Met Gly
         35                  40                  45

Asn Gln Ser Leu Leu Trp Lys Trp Lys Gly Gly Ser Ser Phe Thr Leu
     50                  55                  60

His Lys Lys Leu Ile Val Pro Thr Asp Lys Glu Ala Ser Lys Ala Trp
 65                  70                  75                  80

Gly Arg Ser Ser Thr Pro Val Phe Ser Phe Trp Leu Tyr Asn Glu Lys
                 85                  90                  95

Pro Ile Asp Gly Tyr Leu Thr Ile Asp Phe Gly Glu Lys Leu Ile Ser
            100                 105                 110

Thr Ser Glu Ala Gln Ala Gly Phe Lys Val Lys Leu Asp Phe Thr Gly
        115                 120                 125

Trp Arg Ala Val Gly Val Ser Leu Asn Asn Asp Leu Glu Asn Arg Glu
    130                 135                 140

Met Thr Leu Asn Ala Thr Asn Thr Ser Ser Asp Gly Thr Gln Asp Ser
145                 150                 155                 160

Ile Gly Arg Ser Leu Gly Ala Lys Val Asp Ser Ile Arg Phe Lys Ala
                165                 170                 175
```

```
Pro Ser Asn Val Ser Gln Gly Glu Ile Tyr Ile Asp Arg Ile Met Phe
            180                 185                 190

Ser Val Asp Asp Ala Arg Tyr Gln Trp Ser Asp Tyr Gln Val Lys Thr
        195                 200                 205

Arg Leu Ser Glu Pro Glu Ile Gln Phe His Asn Val Lys Pro Gln Leu
    210                 215                 220

Pro Val Thr Pro Glu Asn Leu Ala Ala Ile Asp Leu Ile Arg Gln Arg
225                 230                 235                 240

Leu Ile Asn Glu Phe Val Gly Gly Glu Lys Glu Thr Asn Leu Ala Leu
                245                 250                 255

Glu Glu Asn Ile Ser Lys Leu Lys Ser Asp Phe Asp Ala Leu Asn Ile
            260                 265                 270

His Thr Leu Ala Asn Gly Gly Thr Gln Gly Arg His Leu Ile Thr Asp
        275                 280                 285

Lys Gln Ile Ile Ile Tyr Gln Pro Glu Asn Leu Asn Ser Gln Asp Lys
    290                 295                 300

Gln Leu Phe Asp Asn Tyr Val Ile Leu Gly Asn Tyr Thr Thr Leu Met
305                 310                 315                 320

Phe Asn Ile Ser Arg Ala Tyr Val Leu Glu Lys Asp Pro Thr Gln Lys
                325                 330                 335

Ala Gln Leu Lys Gln Met Tyr Leu Leu Met Thr Lys His Leu Leu Asp
            340                 345                 350

Gln Gly Phe Val Lys Gly Ser Ala Leu Val Thr Thr His His Trp Gly
        355                 360                 365

Tyr Ser Ser Arg Trp Trp Tyr Ile Ser Thr Leu Leu Met Ser Asp Ala
    370                 375                 380

Leu Lys Glu Ala Asn Leu Gln Thr Gln Val Tyr Asp Ser Leu Leu Trp
385                 390                 395                 400

Tyr Ser Arg Glu Phe Lys Ser Ser Phe Asp Met Lys Val Ser Ala Asp
                405                 410                 415

Ser Ser Asp Leu Asp Tyr Phe Asn Thr Leu Ser Arg Gln His Leu Ala
            420                 425                 430

Leu Leu Leu Leu Glu Pro Asp Asp Gln Lys Arg Ile Asn Leu Val Asn
        435                 440                 445

Thr Phe Ser His Tyr Ile Thr Gly Ala Leu Thr Gln Val Pro Pro Gly
    450                 455                 460

Gly Lys Asp Gly Leu Arg Pro Asp Gly Thr Ala Trp Arg His Glu Gly
465                 470                 475                 480

Asn Tyr Pro Gly Tyr Ser Phe Pro Ala Phe Lys Asn Ala Ser Gln Leu
                485                 490                 495

Ile Tyr Leu Leu Arg Asp Thr Pro Phe Ser Val Gly Glu Ser Gly Trp
            500                 505                 510
```

Asn Asn Leu Lys Lys Ala Met Val Ser Ala Trp Ile Tyr Ser Asn Pro
515 520 525

Glu Val Gly Leu Pro Leu Ala Gly Arg His Pro Phe Asn Ser Pro Ser
530 535 540

Leu Lys Ser Val Ala Gln Gly Tyr Tyr Trp Leu Ala Met Ser Ala Lys
545 550 555 560

Ser Ser Pro Asp Lys Thr Leu Ala Ser Ile Tyr Leu Ala Ile Ser Asp
565 570 575

Lys Thr Gln Asn Glu Ser Thr Ala Ile Phe Gly Glu Thr Ile Thr Pro
580 585 590

Ala Ser Leu Pro Gln Gly Phe Tyr Ala Phe Asn Gly Gly Ala Phe Gly
595 600 605

Ile His Arg Trp Gln Asp Lys Met Val Thr Leu Lys Ala Tyr Asn Thr
610 615 620

Asn Val Trp Ser Ser Glu Ile Tyr Asn Lys Asp Asn Arg Tyr Gly Arg
625 630 635 640

Tyr Gln Ser His Gly Val Ala Gln Ile Val Ser Asn Gly Ser Gln Leu
645 650 655

Ser Gln Gly Tyr Gln Gln Glu Gly Trp Asp Trp Asn Arg Met Gln Gly
660 665 670

Ala Thr Thr Ile His Leu Pro Leu Lys Asp Leu Asp Ser Pro Lys Pro
675 680 685

His Thr Leu Met Gln Arg Gly Glu Arg Gly Phe Ser Gly Thr Ser Ser
690 695 700

Leu Glu Gly Gln Tyr Gly Met Met Ala Phe Asp Leu Ile Tyr Pro Ala
705 710 715 720

Asn Leu Glu Arg Phe Asp Pro Asn Phe Thr Ala Lys Lys Ser Val Leu
725 730 735

Ala Ala Asp Asn His Leu Ile Phe Ile Gly Ser Asn Ile Asn Ser Ser
740 745 750

Asp Lys Asn Lys Asn Val Glu Thr Thr Leu Phe Gln His Ala Ile Thr
755 760 765

Pro Thr Leu Asn Thr Leu Trp Ile Asn Gly Gln Lys Ile Glu Asn Met
770 775 780

Pro Tyr Gln Thr Thr Leu Gln Gln Gly Asp Trp Leu Ile Asp Ser Asn
785 790 795 800

Gly Asn Gly Tyr Leu Ile Thr Gln Ala Glu Lys Val Asn Val Ser Arg
805 810 815

Gln His Gln Val Ser Ala Glu Asn Lys Asn Arg Gln Pro Thr Glu Gly
820 825 830

Asn Phe Ser Ser Ala Trp Ile Asp His Ser Thr Arg Pro Lys Asp Ala
835 840 845

```
Ser Tyr Glu Tyr Met Val Phe Leu Asp Ala Thr Pro Glu Lys Met Gly
    850                 855                 860

Glu Met Ala Gln Lys Phe Arg Glu Asn Asn Gly Leu Tyr Gln Val Leu
865                 870                 875                 880

Arg Lys Asp Lys Asp Val His Ile Ile Leu Asp Lys Leu Ser Asn Val
                885                 890                 895

Thr Gly Tyr Ala Phe Tyr Gln Pro Ala Ser Ile Glu Asp Lys Trp Ile
            900                 905                 910

Lys Lys Val Asn Lys Pro Ala Ile Val Met Thr His Arg Gln Lys Asp
        915                 920                 925

Thr Leu Ile Val Ser Ala Val Thr Pro Asp Leu Asn Met Thr Arg Gln
    930                 935                 940

Lys Ala Ala Thr Pro Val Thr Ile Asn Val Thr Ile Asn Gly Lys Trp
945                 950                 955                 960

Gln Ser Ala Asp Lys Asn Ser Glu Val Lys Tyr Gln Val Ser Gly Asp
                965                 970                 975

Asn Thr Glu Leu Thr Phe Thr Ser Tyr Phe Gly Ile Pro Gln Glu Ile
            980                 985                 990

Lys Leu Ser Pro Leu Pro
        995
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
CAYTTYGCNC ARAAYAAYCC N                                          21
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CACTTCGCNC AAAATAATCC 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CACTTCGCNC AAAACAACCC 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CACTTCGCNC AAAACAATCC 20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CACTTCGCNC AAAATAACCC 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACTTCGCNC AGAATAATCC 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CACTTCGCNC AGAACAACCC 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CACTTCGCNC AGAACAATCC 20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACTTCGCNC AGAATAACCC 20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GARGCNCARG CNGGNTTYAA R 21

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

YTTRAANCCN GCYTGNGCYT C 21

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTGAARCCNG CYTGGGCTTC 20

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTGAARCCNG CYTGAGCTTC 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTGAARCCNG CYTGTGCTTC 20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTGAARCCNG CYTGCGCTTC 20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TTGAARCCNG CYTGGGCCTC 20

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTGAARCCNG CYTGAGCCTC 20

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTGAARCCNG CYTGTGCCTC 20

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTGAARCCNG CYTGCGCCTC 20

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGNGCNAARG TNGAYTCN 18

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGNGCNAARG TNGAYAGY 18

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

NGARTCNACY TTNGCNCC 18

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

RCTRTCNACY TTNGCNCC 18

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GAGTCNACYT TRGCGCC 17

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GAGTCNACYT TRGCACC 17

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GAGTCNACYT TRGCTCC 17

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GAGTCNACYT TRGCCCC 17

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GAGTCNACYT TYGCGCC 17

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GAGTCNACYT TYGCACC 17

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GAGTCNACYT TYGCTCC 17

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GAGTCNACYT TYGCCCC 17

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCCAGCGTTT CTAAGGAGAA AACATATGCC GATATTTCGT TTTACTGC 48

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GCGCCTTATA ACGCGCATAT GGCCACCAGC AATCCTG 37

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6519 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3238..6276

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GGAATTCCAT CACTCAATCA TTAAATTTAG GCACAACGAT GGGCTATCAG CGTTATGACA 60

AATTTAATGA AGGACGCATT GGTTTCACTG TTAGCCAGCG TTTCTAAGGA GAAAAATAAT 120

GCCGATATTT CGTTTTACTG CACTTGCAAT GACATTGGGG CTATTATCAG CGCCTTATAA 180

CGCGATGGCA GCCACCAGCA ATCCTGCATT TGATCCTAAA AATCTGATGC AGTCAGAAAT 240

TTACCATTTT GCACAAAATA ACCCATTAGC AGACTTCTCA TCAGATAAAA ACTCAATACT 300

AACGTTATCT GATAAACGTA GCATTATGGG AAACCAATCT CTTTTATGGA AATGGAAAGG 360

TGGTAGTAGC TTTACTTTAC ATAAAAAACT GATTGTCCCC ACCGATAAAG AAGCATCTAA 420

AGCATGGGGA CGCTCATCTA CCCCCGTTTT CTCATTTTGG CTTTACAATG AAAAACCGAT 480

TGATGGTTAT CTTACTATCG ATTTCGGAGA AAAACTCATT TCAACCAGTG AGGCTCAGGC 540

AGGCTTTAAA GTAAAATTAG ATTTCACTGG CTGGCGTGCT GTGGGAGTCT CTTTAAATAA 600

CGATCTTGAA AATCGAGAGA TGACCTTAAA TGCAACCAAT ACCTCCTCTG ATGGTACTCA 660

AGACAGCATT GGGCGTTCTT TAGGTGCTAA AGTCGATAGT ATTCGTTTTA AAGCGCCTTC 720

TAATGTGAGT CAGGGTGAAA TCTATATCGA CCGTATTATG TTTTCTGTCG ATGATGCTCG 780

CTACCAATGG TCTGATTATC AAGTAAAAAC TCGCTTATCA GAACCTGAAA TTCAATTTCA 840

CAACGTAAAG CCACAACTAC CTGTAACACC TGAAAATTTA GCGGCCATTG ATCTTATTCG 900

CCAACGTCTA ATTAATGAAT TTGTCGGAGG TGAAAAAGAG ACAAACCTCG CATTAGAAGA 960

GAATATCAGC AAATTAAAAA GTGATTTCGA TGCTCTTAAT ATTCACACTT TAGCAAATGG 1020

TGGAACGCAA GGCAGACATC TGATCACTGA TAAACAAATC ATTATTTATC AACCAGAGAA 1080

TCTTAACTCC CAAGATAAAC AACTATTTGA TAATTATGTT ATTTTAGGTA ATTACACGAC 1140

ATTAATGTTT AATATTAGCC GTGCTTATGT GCTGGAAAAA GATCCCACAC AAAAGGCGCA 1200

ACTAAAGCAG ATGTACTTAT TAATGACAAA GCATTTATTA GATCAAGGCT TTGTTAAAGG 1260

GAGTGCTTTA GTGACAACCC ATCACTGGGG ATACAGTTCT CGTTGGTGGT ATATTTCCAC 1320

GTTATTAATG TCTGATGCAC TAAAAGAAGC GAACCTACAA ACTCAAGTTT ATGATTCATT 1380

ACTGTGGTAT TCACGTGAGT TTAAAAGTAG TTTTGATATG AAAGTAAGTG CTGATAGCTC 1440

TGATCTAGAT TATTTCAATA CCTTATCTCG CCAACATTTA GCCTTATTAT TACTAGAGCC 1500

TGATGATCAA AAGCGTATCA ACTTAGTTAA TACTTTCAGC CATTATATCA CTGGCGCATT 1560

AACGCAAGTG CCACCGGGTG GTAAAGATGG TTTACGCCCT GATGGTACAG CATGGCGACA 1620

TGAAGGCAAC TATCCGGGCT ACTCTTTCCC AGCCTTTAAA AATGCCTCTC AGCTTATTTA 1680

TTTATTACGC GATACACCAT TTTCAGTGGG TGAAAGTGGT TGGAATAACC TGAAAAAAGC 1740

GATGGTTTCA GCGTGGATCT ACAGTAATCC AGAAGTTGGA TTACCGCTTG CAGGAAGACA 1800

CCCTTTTAAC TCACCTTCGT TAAAATCAGT CGCTCAAGGC TATTACTGGC TTGCCATGTC 1860

TGCAAAATCA TCGCCTGATA AAACACTTGC ATCTATTTAT CTTGCGATTA GTGATAAAAC 1920

ACAAAATGAA TCAACTGCTA TTTTTGGAGA AACTATTACA CCAGCGTCTT TACCTCAAGG 1980

TTTCTATGCC TTTAATGGCG GTGCTTTTGG TATTCATCGT TGGCAAGATA AAATGGTGAC 2040

ACTGAAAGCT TATAACACCA ATGTTTGGTC ATCTGAAATT TATAACAAAG ATAACCGTTA 2100

TGGCCGTTAC CAAAGTCATG GTGTCGCTCA AATAGTGAGT AATGGCTCGC AGCTTTCACA 2160

GGGCTATCAG CAAGAAGGTT GGGATTGGAA TAGAATGCAA GGGGCAACCA CTATTCACCT 2220

TCCTCTTAAA GACTTAGACA GTCCTAAACC TCATACCTTA ATGCAACGTG GAGAGCGTGG 2280

ATTTAGCGGA ACATCATCCC TTGAAGGTCA ATATGGCATG ATGGCATTCG ATCTTATTTA 2340

TCCCGCCAAT CTTGAGCGTT TTGATCCTAA TTTCACTGCG AAAAAGAGTG TATTAGCCGC 2400

TGATAATCAC TTAATTTTTA TTGGTAGCAA TATAAATAGT AGTGATAAAA ATAAAAATGT 2460

TGAAACGACC TTATTCCAAC ATGCCATTAC TCCAACATTA AATACCCTTT GGATTAATGG 2520

ACAAAAGATA GAAAACATGC CTTATCAAAC AACACTTCAA CAAGGTGATT GGTTAATTGA 2580

TAGCAATGGC AATGGTTACT TAATTACTCA AGCAGAAAAA GTAAATGTAA GTCGCCAACA 2640

TCAGGTTTCA GCGGAAAATA AAAATCGCCA ACCGACAGAA GGAAACTTTA GCTCGGCATG 2700

GATCGATCAC AGCACTCGCC CCAAAGATGC CAGTTATGAG TATATGGTCT TTTTAGATGC 2760

GACACCTGAA AAAATGGGAG AGATGGCACA AAAATTCCGT GAAAATAATG GGTTATATCA 2820

GGTTCTTCGT AAGGATAAAG ACGTTCATAT TATTCTCGAT AAACTCAGCA ATGTAACGGG 2880

ATATGCCTTT TATCAGCCAG CATCAATTGA AGACAAATGG ATCAAAAAGG TTAATAAACC 2940

```
TGCAATTGTG ATGACTCATC GACAAAAAGA CACTCTTATT GTCAGTGCAG TTACACCTGA    3000

TTTAAATATG ACTCGCCAAA AAGCAGCAAC TCCTGTCACC ATCAATGTCA CGATTAATGG    3060

CAAATGGCAA TCTGCTGATA AAAATAGTGA AGTGAAATAT CAGGTTTCTG GTGATAACAC    3120

TGAACTGACG TTTACGAGTT ACTTTGGTAT TCCACAAGAA ATCAAACTCT CGCCACTCCC    3180

TTGATTTAAT CAAAAGAACG CTCTTGCGTT CCTTTTTTAT TTGCAGGAAA TCTGATT       3237

ATG CTA ATA AAA AAC CCT TTA GCC CAC GCG GTT ACA TTA AGC CTC TGT       3285
Met Leu Ile Lys Asn Pro Leu Ala His Ala Val Thr Leu Ser Leu Cys
  1               5                  10                  15

TTA TCA TTA CCC GCA CAA GCA TTA CCC ACT CTG TCT CAT GAA GCT TTC      3333
Leu Ser Leu Pro Ala Gln Ala Leu Pro Thr Leu Ser His Glu Ala Phe
            20                  25                  30

GGC GAT ATT TAT CTT TTT GAA GGT GAA TTA CCC AAT ACC CTT ACC ACT      3381
Gly Asp Ile Tyr Leu Phe Glu Gly Glu Leu Pro Asn Thr Leu Thr Thr
        35                  40                  45

TCA AAT AAT AAT CAA TTA TCG CTA AGC AAA CAG CAT GCT AAA GAT GGT      3429
Ser Asn Asn Asn Gln Leu Ser Leu Ser Lys Gln His Ala Lys Asp Gly
    50                  55                  60

GAA CAA TCA CTC AAA TGG CAA TAT CAA CCA CAA GCA ACA TTA ACA CTA      3477
Glu Gln Ser Leu Lys Trp Gln Tyr Gln Pro Gln Ala Thr Leu Thr Leu
65                  70                  75                  80

AAT AAT ATT GTT AAT TAC CAA GAT GAT AAA AAT ACA GCC ACA CCA CTC      3525
Asn Asn Ile Val Asn Tyr Gln Asp Asp Lys Asn Thr Ala Thr Pro Leu
                85                  90                  95

ACT TTT ATG ATG TGG ATT TAT AAT GAA AAA CCT CAA TCT TCC CCA TTA      3573
Thr Phe Met Met Trp Ile Tyr Asn Glu Lys Pro Gln Ser Ser Pro Leu
            100                 105                 110

ACG TTA GCA TTT AAA CAA AAT AAT AAA ATT GCA CTA AGT TTT AAT GCT      3621
Thr Leu Ala Phe Lys Gln Asn Asn Lys Ile Ala Leu Ser Phe Asn Ala
        115                 120                 125

GAA CTT AAT TTT ACG GGG TGG CGA GGT ATT GCT GTT CCT TTT CGT GAT      3669
Glu Leu Asn Phe Thr Gly Trp Arg Gly Ile Ala Val Pro Phe Arg Asp
    130                 135                 140

ATG CAA GGC TCT GCG ACA GGT CAA CTT GAT CAA TTA GTG ATC ACC GCT      3717
Met Gln Gly Ser Ala Thr Gly Gln Leu Asp Gln Leu Val Ile Thr Ala
145                 150                 155                 160

CCA AAC CAA GCC GGA ACA CTC TTT TTT GAT CAA ATC ATC ATG AGT GTA      3765
Pro Asn Gln Ala Gly Thr Leu Phe Phe Asp Gln Ile Ile Met Ser Val
                165                 170                 175

CCG TTA GAC AAT CGT TGG GCA GTA CCT GAC TAT CAA ACA CCT TAC GTA      3813
Pro Leu Asp Asn Arg Trp Ala Val Pro Asp Tyr Gln Thr Pro Tyr Val
            180                 185                 190

AAT AAC GCA GTA AAC ACG ATG GTT AGT AAA AAC TGG AGT GCA TTA TTG      3861
Asn Asn Ala Val Asn Thr Met Val Ser Lys Asn Trp Ser Ala Leu Leu
        195                 200                 205
```

```
ATG TAC GAT CAG ATG TTT CAA GCC CAT TAC CCT ACT TTA AAC TTC GAT      3909
Met Tyr Asp Gln Met Phe Gln Ala His Tyr Pro Thr Leu Asn Phe Asp
    210                 215                 220

ACT GAA TTT CGC GAT GAC CAA ACA GAA ATG GCT TCG ATT TAT CAG CGC      3957
Thr Glu Phe Arg Asp Asp Gln Thr Glu Met Ala Ser Ile Tyr Gln Arg
225                 230                 235                 240

TTT GAA TAT TAT CAA GGA ATT CGT AGT GAT AAA AAA ATT ACT CCA GAT      4005
Phe Glu Tyr Tyr Gln Gly Ile Arg Ser Asp Lys Lys Ile Thr Pro Asp
                245                 250                 255

ATG CTA GAT AAA CAT TTA GCA TTA TGG GAA AAA TTG GTG TTA ACA CAA      4053
Met Leu Asp Lys His Leu Ala Leu Trp Glu Lys Leu Val Leu Thr Gln
            260                 265                 270

CAC GCT GAT GGC TCA ATC ACA GGA AAA GCC CTT GAT CAC CCT AAC CGG      4101
His Ala Asp Gly Ser Ile Thr Gly Lys Ala Leu Asp His Pro Asn Arg
        275                 280                 285

CAA CAT TTT ATG AAA GTC GAA GGT GTA TTT AGT GAG GGG ACT CAA AAA      4149
Gln His Phe Met Lys Val Glu Gly Val Phe Ser Glu Gly Thr Gln Lys
    290                 295                 300

GCA TTA CTT GAT GCC AAT ATG CTA AGA GAT GTG GGC AAA ACG CTT CTT      4197
Ala Leu Leu Asp Ala Asn Met Leu Arg Asp Val Gly Lys Thr Leu Leu
305                 310                 315                 320

CAA ACT GCT ATT TAC TTG CGT AGC GAT TCA TTA TCA GCA ACT GAT AGA      4245
Gln Thr Ala Ile Tyr Leu Arg Ser Asp Ser Leu Ser Ala Thr Asp Arg
                325                 330                 335

AAA AAA TTA GAA GAG CGC TAT TTA TTA GGT ACT CGT TAT GTC CTT GAA      4293
Lys Lys Leu Glu Glu Arg Tyr Leu Leu Gly Thr Arg Tyr Val Leu Glu
            340                 345                 350

CAA GGT TTT ACA CGA GGA AGT GGT TAT CAA ATT ATT ACT CAT GTT GGT      4341
Gln Gly Phe Thr Arg Gly Ser Gly Tyr Gln Ile Ile Thr His Val Gly
        355                 360                 365

TAC CAA ACC AGA GAA CTT TTT GAT GCA TGG TTT ATT GGC CGT CAT GTT      4389
Tyr Gln Thr Arg Glu Leu Phe Asp Ala Trp Phe Ile Gly Arg His Val
    370                 375                 380

CTT GCA AAA AAT AAC CTT TTA GCC CCC ACT CAA CAA GCT ATG ATG TGG      4437
Leu Ala Lys Asn Asn Leu Leu Ala Pro Thr Gln Gln Ala Met Met Trp
385                 390                 395                 400

TAC AAC GCC ACA GGA CGT ATT TTT GAA AAA AAT AAT GAA ATT GTT GAT      4485
Tyr Asn Ala Thr Gly Arg Ile Phe Glu Lys Asn Asn Glu Ile Val Asp
                405                 410                 415

GCA AAT GTC GAT ATT CTC AAT ACT CAA TTG CAA TGG ATG ATA AAA AGC      4533
Ala Asn Val Asp Ile Leu Asn Thr Gln Leu Gln Trp Met Ile Lys Ser
            420                 425                 430

TTA TTG ATG CTA CCG GAT TAT CAA CAA CGT CAA CAA GCC TTA GCG CAA      4581
Leu Leu Met Leu Pro Asp Tyr Gln Gln Arg Gln Gln Ala Leu Ala Gln
        435                 440                 445

CTG CAA AGT TGG CTA AAT AAA ACC ATT CTA AGC TCA AAA GGT GTT GCT      4629
Leu Gln Ser Trp Leu Asn Lys Thr Ile Leu Ser Ser Lys Gly Val Ala
```

```
    450                 455                 460

GGC GGT TTC AAA TCT GAT GGT TCT ATT TTT CAC CAT TCA CAA CAT TAC      4677
Gly Gly Phe Lys Ser Asp Gly Ser Ile Phe His His Ser Gln His Tyr
465                 470                 475                 480

CCC GCT TAT GCT AAA GAT GCA TTT GGT GGT TTA GCA CCC AGT GTT TAT      4725
Pro Ala Tyr Ala Lys Asp Ala Phe Gly Gly Leu Ala Pro Ser Val Tyr
                485                 490                 495

GCA TTA AGT GAT TCA CCT TTT CGC TTA TCT ACT TCA GCA CAT GAG CGT      4773
Ala Leu Ser Asp Ser Pro Phe Arg Leu Ser Thr Ser Ala His Glu Arg
            500                 505                 510

TTA AAA GAT GTT TTG TTA AAA ATG CGG ATC TAC ACC AAA GAG ACA CAA      4821
Leu Lys Asp Val Leu Leu Lys Met Arg Ile Tyr Thr Lys Glu Thr Gln
        515                 520                 525

ATT CCT GTG GTA TTA AGT GGT CGT CAT CCA ACT GGG TTG CAT AAA ATA      4869
Ile Pro Val Val Leu Ser Gly Arg His Pro Thr Gly Leu His Lys Ile
    530                 535                 540

GGG ATC GCG CCA TTT AAA TGG ATG GCA TTA GCA GGA ACC CCA GAT GGC      4917
Gly Ile Ala Pro Phe Lys Trp Met Ala Leu Ala Gly Thr Pro Asp Gly
545                 550                 555                 560

AAA CAA AAG TTA GAT ACC ACA TTA TCC GCC GCT TAT GCA AAA TTA GAC      4965
Lys Gln Lys Leu Asp Thr Thr Leu Ser Ala Ala Tyr Ala Lys Leu Asp
                565                 570                 575

AAC AAA ACG CAT TTT GAA GGC ATT AAC GCT GAA AGT GAG CCA GTC GGC      5013
Asn Lys Thr His Phe Glu Gly Ile Asn Ala Glu Ser Glu Pro Val Gly
            580                 585                 590

GCA TGG GCA ATG AAT TAT GCA TCA ATG GCA ATA CAA CGA AGA GCA TCG      5061
Ala Trp Ala Met Asn Tyr Ala Ser Met Ala Ile Gln Arg Arg Ala Ser
        595                 600                 605

ACC CAA TCA CCA CAA CAA AGC TGG CTC GCC ATA GCG CGC GGT TTT AGC      5109
Thr Gln Ser Pro Gln Gln Ser Trp Leu Ala Ile Ala Arg Gly Phe Ser
    610                 615                 620

CGT TAT CTT GTT GGT AAT GAA AGC TAT GAA AAT AAC AAC CGT TAT GGT      5157
Arg Tyr Leu Val Gly Asn Glu Ser Tyr Glu Asn Asn Asn Arg Tyr Gly
625                 630                 635                 640

CGT TAT TTA CAA TAT GGA CAA TTG GAA ATT ATT CCA GCT GAT TTA ACT      5205
Arg Tyr Leu Gln Tyr Gly Gln Leu Glu Ile Ile Pro Ala Asp Leu Thr
                645                 650                 655

CAA TCA GGG TTT AGC CAT GCT GGA TGG GAT TGG AAT AGA TAT CCA GGT      5253
Gln Ser Gly Phe Ser His Ala Gly Trp Asp Trp Asn Arg Tyr Pro Gly
            660                 665                 670

ACA ACA ACT ATT CAT CTT CCC TAT AAC GAA CTT GAA GCA AAA CTT AAT      5301
Thr Thr Thr Ile His Leu Pro Tyr Asn Glu Leu Glu Ala Lys Leu Asn
        675                 680                 685

CAA TTA CCT GCT GCA GGT ATT GAA GAA ATG TTG CTT TCA ACA GAA AGT      5349
Gln Leu Pro Ala Ala Gly Ile Glu Glu Met Leu Leu Ser Thr Glu Ser
    690                 695                 700
```

```
TAC TCT GGT GCA AAT ACC CTT AAT AAT AAC AGT ATG TTT GCC ATG AAA      5397
Tyr Ser Gly Ala Asn Thr Leu Asn Asn Asn Ser Met Phe Ala Met Lys
705                 710                 715                 720

TTA CAC GGT CAC AGT AAA TAT CAA CAA CAA AGC TTA AGG GCA AAT AAA      5445
Leu His Gly His Ser Lys Tyr Gln Gln Gln Ser Leu Arg Ala Asn Lys
                725                 730                 735

TCC TAT TTC TTA TTT GAT AAT AGA GTT ATT GCT TTA GGC TCA GGT ATT      5493
Ser Tyr Phe Leu Phe Asp Asn Arg Val Ile Ala Leu Gly Ser Gly Ile
            740                 745                 750

GAA AAT GAT GAT AAA CAA CAT ACG ACC GAA ACA ACA CTA TTC CAG TTT      5541
Glu Asn Asp Asp Lys Gln His Thr Thr Glu Thr Thr Leu Phe Gln Phe
        755                 760                 765

GCC GTC CCT AAA TTA CAG TCA GTG ATC ATT AAT GGC AAA AAG GTA AAT      5589
Ala Val Pro Lys Leu Gln Ser Val Ile Ile Asn Gly Lys Lys Val Asn
    770                 775                 780

CAA TTA GAT ACT CAA TTA ACT TTA AAT AAT GCA GAT ACA TTA ATT GAT      5637
Gln Leu Asp Thr Gln Leu Thr Leu Asn Asn Ala Asp Thr Leu Ile Asp
785                 790                 795                 800

CCT GCC GGC AAT TTA TAT AAG CTC ACT AAA GGA CAA ACT GTA AAA TTT      5685
Pro Ala Gly Asn Leu Tyr Lys Leu Thr Lys Gly Gln Thr Val Lys Phe
                805                 810                 815

AGT TAT CAA AAA CAA CAT TCA CTT GAT GAT AGA AAT TCA AAA CCA ACA      5733
Ser Tyr Gln Lys Gln His Ser Leu Asp Asp Arg Asn Ser Lys Pro Thr
            820                 825                 830

GAA CAA TTA TTT GCA ACA GCT GTT ATT TCT CAT GGT AAG GCA CCG AGT      5781
Glu Gln Leu Phe Ala Thr Ala Val Ile Ser His Gly Lys Ala Pro Ser
        835                 840                 845

AAT GAA AAT TAT GAA TAT GCA ATA GCT ATC GAA GCA CAA AAT AAT AAA      5829
Asn Glu Asn Tyr Glu Tyr Ala Ile Ala Ile Glu Ala Gln Asn Asn Lys
    850                 855                 860

GCT CCC GAA TAC ACA GTA TTA CAA CAT AAT GAT CAG CTC CAT GCG GTA      5877
Ala Pro Glu Tyr Thr Val Leu Gln His Asn Asp Gln Leu His Ala Val
865                 870                 875                 880

AAA GAT AAA ATA ACC CAA GAA GAG GGA TAT GCT TTT TTT GAA GCC ACT      5925
Lys Asp Lys Ile Thr Gln Glu Glu Gly Tyr Ala Phe Phe Glu Ala Thr
                885                 890                 895

AAG TTA AAA TCA GCG GAT GCA ACA TTA TTA TCC AGT GAT GCG CCG GTT      5973
Lys Leu Lys Ser Ala Asp Ala Thr Leu Leu Ser Ser Asp Ala Pro Val
            900                 905                 910

ATG GTC ATG GCT AAA ATA CAA AAT CAG CAA TTA ACA TTA AGT ATT GTT      6021
Met Val Met Ala Lys Ile Gln Asn Gln Gln Leu Thr Leu Ser Ile Val
        915                 920                 925

AAT CCT GAT TTA AAT TTA TAT CAA GGT AGA GAA AAA GAT CAA TTT GAT      6069
Asn Pro Asp Leu Asn Leu Tyr Gln Gly Arg Glu Lys Asp Gln Phe Asp
    930                 935                 940

GAT AAA GGT AAT CAA ATC GAA GTT AGT GTT TAT TCT CGT CAT TGG CTT      6117
Asp Lys Gly Asn Gln Ile Glu Val Ser Val Tyr Ser Arg His Trp Leu
```

```
945                 950                 955                 960

ACA GCA GAA TCG CAA TCA ACA AAT AGT ACT ATT ACC GTA AAA GGA ATA      6165
Thr Ala Glu Ser Gln Ser Thr Asn Ser Thr Ile Thr Val Lys Gly Ile
                965                 970                 975

TGG AAA TTA ACG ACA CCT CAA CCC GGT GTT ATT ATT AAG CAC CAC AAT      6213
Trp Lys Leu Thr Thr Pro Gln Pro Gly Val Ile Ile Lys His His Asn
            980                 985                 990

AAC AAC ACT CTT ATT ACG ACA ACA ACC ATA CAG GCA ACA CCT ACT GTT      6261
Asn Asn Thr Leu Ile Thr Thr Thr Thr Ile Gln Ala Thr Pro Thr Val
        995                 1000                1005

ATT AAT TTA GTT AAG TAAATTTCGT AACTTTTAAA CTAAAGAGTC TCGACATAAA      6316
Ile Asn Leu Val Lys
    1010

AATATCGAGA CTCTTTTTAT TAAAAAATTA AAAACAAGTT AACGAATGAA TTAATTATTT    6376

GAAAAATAAA AAATAAATCG ATAGCTTTAT TATTGATAAT AAATGTGTTG TGCTCAATGG    6436

TTATTTTGTT ATTCTCTGCG CGGATGCTTG GATCAATCTG GTTCAAGCAT ATCGCAAGCA    6496

CCAGAACGAA AAAAGCCCCG GGT                                            6519
```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1013 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
Met Leu Ile Lys Asn Pro Leu Ala His Ala Val Thr Leu Ser Leu Cys
  1               5                  10                  15

Leu Ser Leu Pro Ala Gln Ala Leu Pro Thr Leu Ser His Glu Ala Phe
            20                  25                  30

Gly Asp Ile Tyr Leu Phe Glu Gly Glu Leu Pro Asn Thr Leu Thr Thr
        35                  40                  45

Ser Asn Asn Asn Gln Leu Ser Leu Ser Lys Gln His Ala Lys Asp Gly
    50                  55                  60

Glu Gln Ser Leu Lys Trp Gln Tyr Gln Pro Gln Ala Thr Leu Thr Leu
65                  70                  75                  80

Asn Asn Ile Val Asn Tyr Gln Asp Asp Lys Asn Thr Ala Thr Pro Leu
                85                  90                  95

Thr Phe Met Met Trp Ile Tyr Asn Glu Lys Pro Gln Ser Ser Pro Leu
            100                 105                 110

Thr Leu Ala Phe Lys Gln Asn Asn Lys Ile Ala Leu Ser Phe Asn Ala
        115                 120                 125
```

```
Glu Leu Asn Phe Thr Gly Trp Arg Gly Ile Ala Val Pro Phe Arg Asp
    130                 135                 140

Met Gln Gly Ser Ala Thr Gly Gln Leu Asp Gln Leu Val Ile Thr Ala
145                 150                 155                 160

Pro Asn Gln Ala Gly Thr Leu Phe Phe Asp Gln Ile Ile Met Ser Val
                165                 170                 175

Pro Leu Asp Asn Arg Trp Ala Val Pro Asp Tyr Gln Thr Pro Tyr Val
            180                 185                 190

Asn Asn Ala Val Asn Thr Met Val Ser Lys Asn Trp Ser Ala Leu Leu
        195                 200                 205

Met Tyr Asp Gln Met Phe Gln Ala His Tyr Pro Thr Leu Asn Phe Asp
    210                 215                 220

Thr Glu Phe Arg Asp Asp Gln Thr Glu Met Ala Ser Ile Tyr Gln Arg
225                 230                 235                 240

Phe Glu Tyr Tyr Gln Gly Ile Arg Ser Asp Lys Lys Ile Thr Pro Asp
                245                 250                 255

Met Leu Asp Lys His Leu Ala Leu Trp Glu Lys Leu Val Leu Thr Gln
            260                 265                 270

His Ala Asp Gly Ser Ile Thr Gly Lys Ala Leu Asp His Pro Asn Arg
        275                 280                 285

Gln His Phe Met Lys Val Glu Gly Val Phe Ser Glu Gly Thr Gln Lys
    290                 295                 300

Ala Leu Leu Asp Ala Asn Met Leu Arg Asp Val Gly Lys Thr Leu Leu
305                 310                 315                 320

Gln Thr Ala Ile Tyr Leu Arg Ser Asp Ser Leu Ser Ala Thr Asp Arg
                325                 330                 335

Lys Lys Leu Glu Glu Arg Tyr Leu Leu Gly Thr Arg Tyr Val Leu Glu
            340                 345                 350

Gln Gly Phe Thr Arg Gly Ser Gly Tyr Gln Ile Ile Thr His Val Gly
        355                 360                 365

Tyr Gln Thr Arg Glu Leu Phe Asp Ala Trp Phe Ile Gly Arg His Val
    370                 375                 380

Leu Ala Lys Asn Asn Leu Leu Ala Pro Thr Gln Gln Ala Met Met Trp
385                 390                 395                 400

Tyr Asn Ala Thr Gly Arg Ile Phe Glu Lys Asn Asn Glu Ile Val Asp
                405                 410                 415

Ala Asn Val Asp Ile Leu Asn Thr Gln Leu Gln Trp Met Ile Lys Ser
            420                 425                 430

Leu Leu Met Leu Pro Asp Tyr Gln Gln Arg Gln Gln Ala Leu Ala Gln
        435                 440                 445

Leu Gln Ser Trp Leu Asn Lys Thr Ile Leu Ser Ser Lys Gly Val Ala
    450                 455                 460
```

Gly Gly Phe Lys Ser Asp Gly Ser Ile Phe His His Ser Gln His Tyr
465 470 475 480

Pro Ala Tyr Ala Lys Asp Ala Phe Gly Gly Leu Ala Pro Ser Val Tyr
485 490 495

Ala Leu Ser Asp Ser Pro Phe Arg Leu Ser Thr Ser Ala His Glu Arg
500 505 510

Leu Lys Asp Val Leu Leu Lys Met Arg Ile Tyr Thr Lys Glu Thr Gln
515 520 525

Ile Pro Val Val Leu Ser Gly Arg His Pro Thr Gly Leu His Lys Ile
530 535 540

Gly Ile Ala Pro Phe Lys Trp Met Ala Leu Ala Gly Thr Pro Asp Gly
545 550 555 560

Lys Gln Lys Leu Asp Thr Thr Leu Ser Ala Ala Tyr Ala Lys Leu Asp
565 570 575

Asn Lys Thr His Phe Glu Gly Ile Asn Ala Glu Ser Glu Pro Val Gly
580 585 590

Ala Trp Ala Met Asn Tyr Ala Ser Met Ala Ile Gln Arg Arg Ala Ser
595 600 605

Thr Gln Ser Pro Gln Gln Ser Trp Leu Ala Ile Ala Arg Gly Phe Ser
610 615 620

Arg Tyr Leu Val Gly Asn Glu Ser Tyr Glu Asn Asn Asn Arg Tyr Gly
625 630 635 640

Arg Tyr Leu Gln Tyr Gly Gln Leu Glu Ile Ile Pro Ala Asp Leu Thr
645 650 655

Gln Ser Gly Phe Ser His Ala Gly Trp Asp Trp Asn Arg Tyr Pro Gly
660 665 670

Thr Thr Thr Ile His Leu Pro Tyr Asn Glu Leu Glu Ala Lys Leu Asn
675 680 685

Gln Leu Pro Ala Ala Gly Ile Glu Glu Met Leu Leu Ser Thr Glu Ser
690 695 700

Tyr Ser Gly Ala Asn Thr Leu Asn Asn Asn Ser Met Phe Ala Met Lys
705 710 715 720

Leu His Gly His Ser Lys Tyr Gln Gln Gln Ser Leu Arg Ala Asn Lys
725 730 735

Ser Tyr Phe Leu Phe Asp Asn Arg Val Ile Ala Leu Gly Ser Gly Ile
740 745 750

Glu Asn Asp Asp Lys Gln His Thr Thr Glu Thr Thr Leu Phe Gln Phe
755 760 765

Ala Val Pro Lys Leu Gln Ser Val Ile Ile Asn Gly Lys Lys Val Asn
770 775 780

Gln Leu Asp Thr Gln Leu Thr Leu Asn Asn Ala Asp Thr Leu Ile Asp
785 790 795 800

```
Pro Ala Gly Asn Leu Tyr Lys Leu Thr Lys Gly Gln Thr Val Lys Phe
                805                 810                 815

Ser Tyr Gln Lys Gln His Ser Leu Asp Asp Arg Asn Ser Lys Pro Thr
            820                 825                 830

Glu Gln Leu Phe Ala Thr Ala Val Ile Ser His Gly Lys Ala Pro Ser
        835                 840                 845

Asn Glu Asn Tyr Glu Tyr Ala Ile Ala Ile Glu Ala Gln Asn Asn Lys
    850                 855                 860

Ala Pro Glu Tyr Thr Val Leu Gln His Asn Asp Gln Leu His Ala Val
865                 870                 875                 880

Lys Asp Lys Ile Thr Gln Glu Glu Gly Tyr Ala Phe Phe Glu Ala Thr
                885                 890                 895

Lys Leu Lys Ser Ala Asp Ala Thr Leu Leu Ser Ser Asp Ala Pro Val
            900                 905                 910

Met Val Met Ala Lys Ile Gln Asn Gln Gln Leu Thr Leu Ser Ile Val
        915                 920                 925

Asn Pro Asp Leu Asn Leu Tyr Gln Gly Arg Glu Lys Asp Gln Phe Asp
    930                 935                 940

Asp Lys Gly Asn Gln Ile Glu Val Ser Val Tyr Ser Arg His Trp Leu
945                 950                 955                 960

Thr Ala Glu Ser Gln Ser Thr Asn Ser Thr Ile Thr Val Lys Gly Ile
                965                 970                 975

Trp Lys Leu Thr Thr Pro Gln Pro Gly Val Ile Ile Lys His His Asn
            980                 985                 990

Asn Asn Thr Leu Ile Thr Thr Thr Thr Ile Gln Ala Thr Pro Thr Val
        995                 1000                1005

Ile Asn Leu Val Lys
    1010
```

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

ATTTGCAGGA AATCTGCATA TGCTAATAAA AAACCC 36

What is claimed is:

1. A purified isolated DNA fragment of Proteus vulgaris (P. vulgaris) comprising a sequence encoding for the chondroitinase I enzyme.

2. A purified isolated DNA fragment of P. vulgaris, wherein the fragment comprises a sequence which hybridizes with a nucleic acid sequence encoding for the amino acids numbered 1-1021 of SEQ ID NO:2 or a biological equivalent thereof.

3. The purified isolated DNA fragment of Claim 2, wherein the fragment has the sequence of (a) the nucleotides numbered 119-3181 of SEQ ID NO:1, or (b) the nucleotides numbered 119-3181 of SEQ ID NO:3.

4. A purified isolated DNA fragment of P. vulgaris, wherein the fragment comprises a sequence which hybridizes with a nucleic acid sequence encoding for the amino acids numbered 25-1021 of SEQ ID NO:2 or a biological equivalent thereof.

5. The purified isolated DNA fragment of Claim 4, wherein the fragment has the sequence of (a) the nucleotides numbered 191-3181 of SEQ ID NO:1, or (b) the nucleotides numbered 191-3181 of SEQ ID NO:3.

6. A purified isolated DNA fragment of P. vulgaris, wherein the fragment comprises a sequence which hybridizes with a nucleic acid sequence encoding for the amino acids numbered 24-1021 of SEQ ID NO:5 or a biological equivalent thereof.

7. The purified isolated DNA fragment of Claim 6, wherein the fragment has the sequence of nucleotides numbered 188-3181 of SEQ ID NO:4.

8. A purified isolated DNA fragment of Proteus vulgaris (P. vulgaris) comprising a sequence encoding for chondroitinase II enzyme.

9. A purified isolated DNA fragment of P.

vulgaris, wherein the fragment comprises a sequence which hybridizes with a nucleic acid sequence encoding for (a) the amino acids numbered 1-1013 of SEQ ID NO:40 or a biological equivalent thereof, or (b) the amino acids numbered 24-1013 of SEQ ID NO:40 or a biological equivalent thereof.

10. The purified isolated DNA fragment of Claim 9, wherein the fragment has the sequence of nucleotides (a) numbered 3238-6276 of SEQ ID NO:39, or (b) numbered 3307-6276 of SEQ ID NO:39.

11. A plasmid containing a purified isolated DNA fragment of P. vulgaris comprising the sequence of (a) Claim 1 or (b) Claim 8.

12. The plasmid of Claim 11 wherein the plasmid is that designated pTM49-6 or that designated LP^{2}1359.

13. A host cell transformed with the plasmid of Claim 11.

14. The host cell of Claim 13 wherein the plasmid is that designated pTM49-6 (ATCC 69234) or that designated LP^{2}1359 (ATCC 69598).

15. A purified isolated recombinant chondroitinase I enzyme.

16. The chondroitinase I enzyme of Claim 15, whose amino acid sequence is depicted for (a) the amino acids numbered 1-1021 of SEQ ID NO:2 or a biological equivalent thereof, (b) the amino acids numbered 25-1021 of SEQ ID NO:2 or a biological equivalent thereof, or (c) the amino acids numbered 24-1021 of SEQ ID NO:5 or a biological equivalent thereof.

17. A purified isolated recombinant chondroitinase II enzyme.

18. The chondroitinase II enzyme of Claim 17, whose amino acid sequence is depicted for (a) the

amino acids numbered 1-1013 of SEQ ID NO:40 or a biological equivalent thereof, or (b) the amino acids numbered 24-1013 of SEQ ID NO:40 or a biological equivalent thereof.

19. A method of producing chondroitinase I enzyme which comprises transforming a host cell with the plasmid of Claim 11(a) and culturing the host cell under conditions which permit expression of said enzyme by the host cell.

20. A method of producing the chondroitinase II enzyme which comprises transforming a host cell with the plasmid of Claim 11(b) and culturing the host cell under conditions which permit expression of said enzyme by the host cell.

21. A method for the isolation and purification of the recombinant chondroitinase I enzyme of Proteus vulgaris from host cells, said method comprising the steps of:

(a) lysing by homogenization the host cells to release the enzyme into the supernatant;

(b) subjecting the supernatant to diafiltration to remove salts and other small molecules;

(c) passing the supernatant through an anion exchange resin-containing column;

(d) loading the eluate from step (c) to a cation exchange resin-containing column so that the enzyme in the eluate binds to the cation exchange column; and

(e) eluting the enzyme bound to the cation exchange column with a solvent capable of releasing the enzyme from the column.

22. The method of Claim 21, wherein prior

to step (b), the following two steps are performed:

(1) treating the supernatant with an acidic solution to precipitate out the enzyme; and

(2) recovering the pellet and then dissolving it in an alkali solution to again place the enzyme in a basic environment.

23. A recombinant chondroitinase I enzyme isolated and purified by the method of Claim 21 or by the method of Claim 22.

24. A method for the isolation and purification of the recombinant chondroitinase II enzyme of Proteus vulgaris from host cells, said method comprising the steps of:

(a) lysing by homogenization the host cells to release the enzyme into the supernatant;

(b) subjecting the supernatant to diafiltration to remove salts and other small molecules;

(c) passing the supernatant through an anion exchange resin-containing column;

(d) loading the eluate from step (c) to a cation exchange resin-containing column so that the enzyme in the eluate binds to the cation exchange column; and

(e) obtaining by affinity elution the enzyme bound to the cation exchange column with a solution of chondroitin sulfate, such that the enzyme is co-eluted with the chondroitin sulfate;

(f) loading the eluate from step (e) to an anion exchange resin-containing column and eluting the enzyme with a solvent

such that the chondroitin sulfate binds to the column; and

(g) concentrating the eluate from step (f) and crystallizing out the enzyme from the supernatant which contains an approximately 37 kD contaminant.

25. The method of Claim 24, wherein prior to step (b), the following two steps are performed:

(1) treating the supernatant with an acidic solution to precipitate out the enzyme; and

(2) recovering the pellet and then dissolving it in an alkali solution to again place the enzyme in a basic environment.

26. A recombinant chondroitinase II enzyme isolated and purified by the method of Claim 24 or by the method of Claim 25.

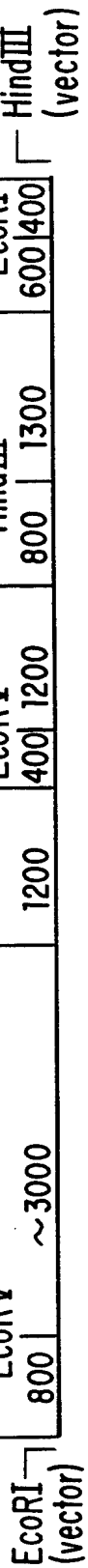

EcoRI (vector)
NsiI
EcoRV
800
~3000
BstYI
1200
EcoRV
400
1200
BstYI
800
HindIII
1300
HindIII
600
EcoRI
400
NsiI
HindIII (vector)

FIG. 1

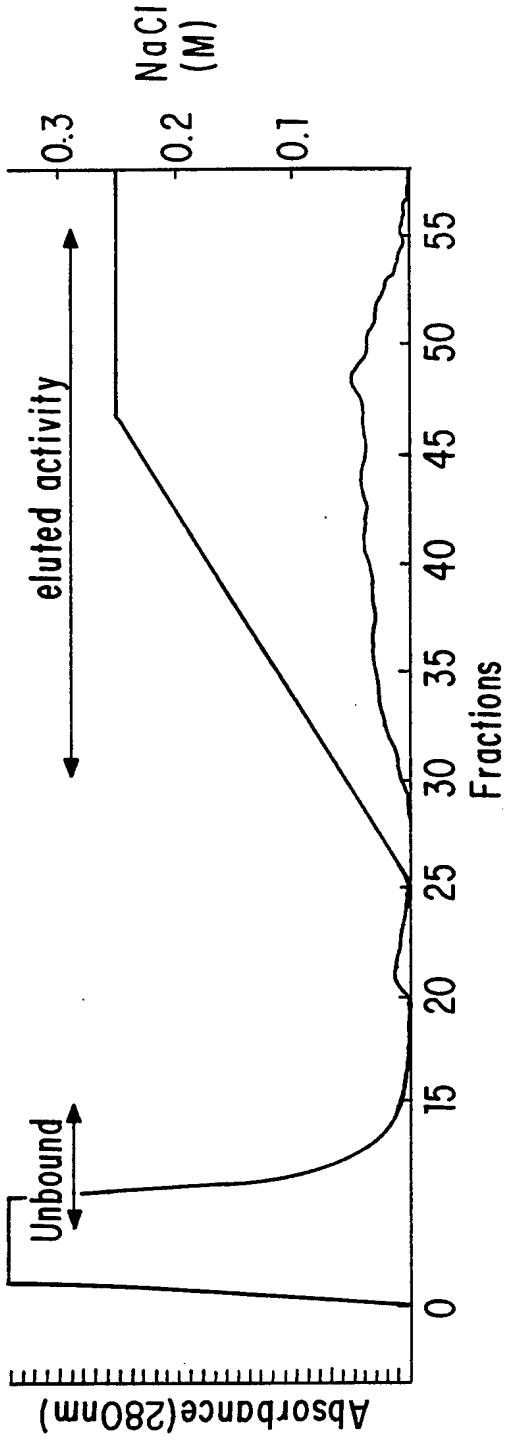

NaCl (M)
0.3
0.2
0.1
eluted activity
Unbound
0
15
20
25
30
35
40
45
50
55
Fractions
Absorbance(280nm)

FIG. 2

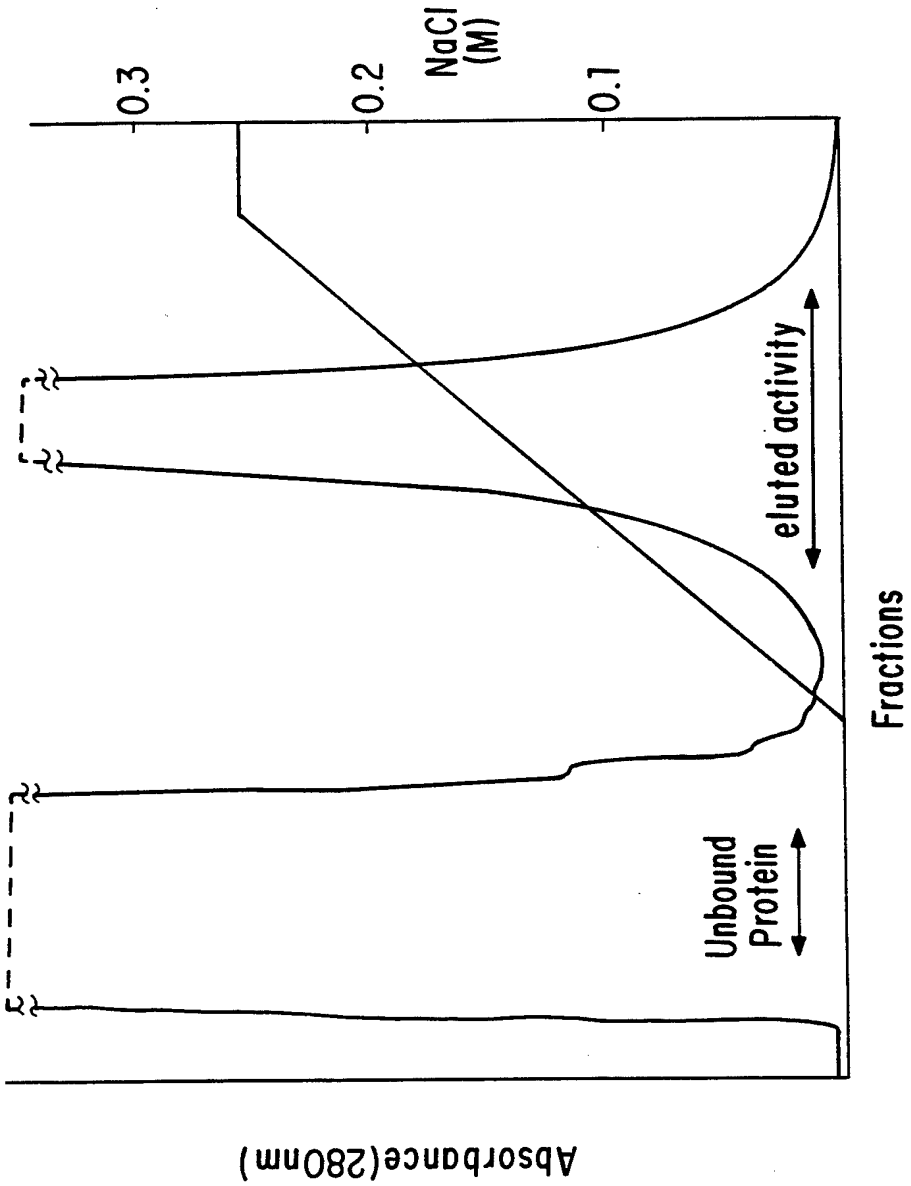

NaCl (M)
0.3
0.2
0.1
eluted activity
Unbound Protein
Fractions
Absorbance(280nm)

FIG. 3

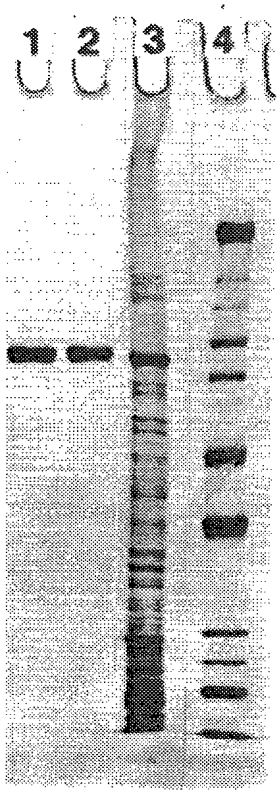


FIG. 4

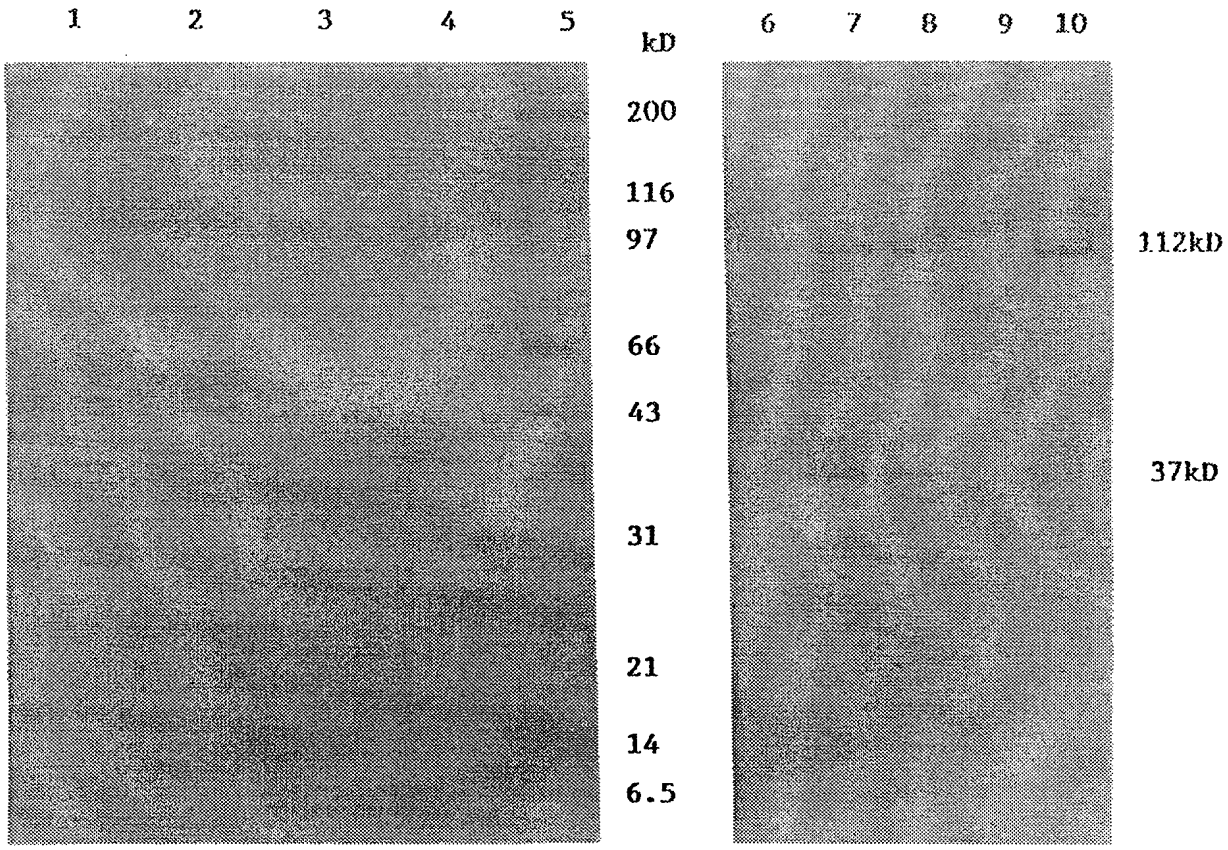


FIG. 5A

FIG. 5B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04495

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 1/21, 9/88, 15/31, 15/60, 15/61
US CL :435/69.1, 232, 252.3, 320.1; 536/23.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 232, 252.3, 320.1; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, BIOSIS, INPADOC, JICST-E search terms: chondroitinase, Proteus vulgaris

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US, A, 5,049,501 (KATSURAGI et al) 17 September 1991, column 4, line 1 to column 5, line 68.	1,2,4,6,8, 9 ,11,13 ---------- 3,5,7,10, 12,14,19, 2 0,2 1-26
X ---- Y	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, Volume 50, Number 4, issued 1986, Sato et al, "Subunit Structure of Chondroitinase ABC from Proteus vulgaris", pages 1057-1059, see page 1057, paragraph bridging columns 1-2.	15-18 ---------- 21-26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
01 JULY 1994	JUL 15 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer STEPHEN WALSH Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Int .ional application No.
PCT/US94/04495

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 243, Number 7, issued 10 April 1968, Yamagata et al, "Purification and Properties of Bacterial Chondroitinases and Chondrosulfatases", pages 1523-1535, see Purification section, pages 1526-1527.	15-18 ---------- 21-26
Y	US, A, 5,198,355 (KIKUCHI et al) 30 March 1993, see column 3, lines 3-25.	21-26
Y	D. M. GLOVER, "DNA CLONING, VOLUME 1, A PRACTICAL APPROACH", published 1985 by IRL Press (Oxford), pages 49-77, see page 49, paragraph 1.	11-14,19, 2 0

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US94/04495

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Int ional application No.
PCT/US94/04495

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-7, 11-16, 19 and 21-23, directed to DNA encoding chondroitinase I, plasmids, host cells, chondroitinase I, and a method for purifying chondroitinase I, classified in Class 435, subclass 232.

II. Claims 8-14, 17, 18, 20 and 24-26, directed to DNA encoding chondroitinase II, plasmids, host cells, chondroitinase II, and a method for purifying chondroitinase II, classified in Class 435, subclass 232.

Claims 11-14 link the groups because they include both species of chondroitinase. In the event the additional fee is not paid, claims 11-14 will be searched to the extent they embrace chondroitinase I.

The claims are directed to two distinct species of enzyme which are not so linked by a special technical feature so as to form a single general inventive concept as required by PCT Rules 13.1-13.3. The special technical features of the two species are disclosed as distinguishing them from each other and from the prior art.